University of Alberta

ADRENOMEDULLIN REGULATES NEUROENDOCRINE AND CENTRAL AUTONOMIC FUNCTIONS

By

Jing Shan ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Cell Biology

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ABSTRACT

Studies were designed to study the role of adrenomedullin (ADM) in regulating autonomic and neuroendocrine functions. (1) Intravenous ADM activates neurons in the hypothalamic paraventricular nucleus (PVN), brainstem, and area postrema (AP), a circumventricular organ which lacks a blood-brain barrier. Furthermore, AP ablation attenuates activation in the PVN, providing evidence that systemic ADM communicates with the AP to affect brain functions. (2) Expression of the ADM gene in autonomic centers and its downregulation in response to different stressors suggest that ADM produced in the brain influences autonomic responses and activity of the hypothalamo-pituitary-adrenal (HPA) axis. (3) Stimulatory effects of intracerebroventricular ADM on arterial pressure, plasma adrenocorticotrophic hormone, and nitric oxide production in the hypothalamus indicate that central ADM stimulates the sympathetic nervous system and HPA axis, and that hypothalamic NO may be involved. Together, the results support the hypothesis that central ADM plays an important role in maintaining body homeostasis.

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Adrenomedullin Regulates Neuroendocrine and Central Autonomic Functions** by Jing Shan in partial fulfillment of the requirements for the degree of Master of Science.

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TABLE OF CONTENTS

Chapter 1: Introduction 1

1.1 Overview 2

1.2 Adrenomedullin 3

1.2.1 Discovery and structure 3

1.2.2 Distribution 4

1.2.3 Regulation of production and secretion 5

1.2.4 Receptors and signal transduction 6

1.2.5 Biological functions 7

1.3 *c-fos* acts as a marker of activated neurons 8

1.4 Involvement of nitric oxide 9

1.5 Hypotheses 10

1.6 References 12

Chapter 2: Area postrema ablation attenuates activation of neurons in the paraventricular nucleus in response to systemic adrenomedullin 22

2.1 Abstract 23

2.2 Introduction 24

2.3 Materials and methods 26

2.3.1 Animals 26

2.3.2 Experiment protocols 26

2.3.2.1 Experiment 1 26

2.3.2.2 Experiment 2 27

2.3.2.3 Experiment 3 27

2.3.3 Processing of tissues 28

2.3.4 Subdivision of nuclei 29

2.3.5 Analysis 29

2.4 Results 30

2.4.1 Experiment 1: Activation of neurons in autonomic centers in response to iv administration of ADM or NP. 30

2.4.1.1 Mean arterial pressure 30

2.4.1.2 Neuronal labeling in the PVN 30

2.1.4.3 Neurons with FLI in the NTS and CVOs 31

2.4.2 Experiment 2: Activation of PVN neurons in AP-A rats or AP-S rats 31

2.4.3 Experiment 3: Activation of PVN neurons in AP-A rats or AP-S rats in response to iv administration of ADM 31

2.4.3.1 Mean arterial pressure 31

2.4.3.2 Neuronal labeling in the PVN 32

2.5 Discussion 32

2.6 References 37

Chapter 3: The distribution of preproadrenomedullin mRNA in the rat central nervous system and its modulation by physiological stressors 48

3.1 Abstract 49

3.2 Introduction 50

3.3 Materials and methods 51

3.3.1 Animals 51

3.3.2 Experimental protocol 52

3.3.2.1 ADM mRNA survey 52

3.3.2.2 Effects of LPS on Fos expression and ADM mRNA expression 52

3.3.2.3 Effects of restraint stress on ADM mRNA expression 52

3.3.2.4 Effects of dehydration on ADM mRNA expression 53

3.3.3 In situ hybridization (ISH) 53

3.3.4 Combined Fos immunohistochemistry and ISH 54

3.3.5 Production of photomicrographs 55

3.3.6 Analysis 55

3.4 Results 56

3.4.1 Distribution of ppADM mRNA in the rat CNS 56

3.4.2 Effects of LPS on FLI and ppADM gene expression 58

3.4.3 Effects of restraint stress on ppADM gene expression 58

3.4.4 Effects of dehydration on ppADM gene expression 59

3.5 Discussion 59

3.5.1 Distribution of ppADM mRNA in the rat CNS 59

3.5.2 Modulation of ppADM mRNA gene expression in response to physiological stressors 62

3.5.3 Conclusions 66

3.6 References 68

Chapter 4: Stimulatory effects of intracerebroventricular ADM on the hypothalamo-pituitary-adrenal axis and the sympathetic nervous system 83

4.1 Abstract 84

4.2 Introduction 85

4.3 Materials and methods 87

4.3.1 Animals 87

4.3.2 Surgical procedures 87

4.3.3 Experimental design 88

4.3.4 Plasma ACTH assay 89

4.3.5 Nitrate/Nitrite colorimetric assay 90

4.3.6 Fos immunohistochemistry 90

4.3.7 NADPH-d histochemistry 91

4.3.8 In situ hybridization for CRF, AVP, OXY and TH mRNA 91

4.3.9 Production of photomicrographs 93

4.3.10 Subdivision of nuclei 93

4.3.11 Analysis 94

4.4 Results 95

4.4.1 Effects of icv injections of ADM on MAP 95

4.4.2 Effects of icv injections of ADM on plasma ACTH 95

4.4.3 Effects of icv injections of ADM on NO production in hypothalamus and brainstem 95

4.4.4 Effects of icv injections of ADM on neurons labeled for FLI 96

4.4.5 Effects of icv injections of ADM on mRNA expression for CRF, OXY, AVP, and TH 96

4.5 Discussion 97

4.5.1 Stimulatory effects on the HPA axis 97

4.5.2 Stimulatory effects on the sympathetic nervous system 99

4.5.3 Effects on the hypothalamo-neurohypophysial system 101

4.5.4 The involvement of NO in these responses 103

4.5.5 Conclusion 104

4.6 References 105

Chapter 5: General discussion and conclusions 115

5.1 Discussion and conclusions 116

5.2 References 120

LIST OF TABLES

Table 1-1	Distribution of ADM-like immunoreactivity	19
Table 1-2	Multiple actions of ADM gene-derived peptides	20
Table 3-1	Distribution and relative quantity of ppADM mRNA expressing neurons in the rat brain.	75

LIST OF FIGURES

Figure 1-1	Processing of the ADM gene.	21
Figure 2-1	Changes in MAP of rats after ADM injections or NP infusions.	40
Figure 2-2	Photographs showing FLI and NADPH-d labeled neurons in the PVN, AP, and NTS from ADM rats or NP rats.	41
Figure 2-3	Numbers of neurons labeled for FLI and/or NADPH-d in rostral, middle, and caudal regions of the PVN (left), and four subdivisions of the middle PVN (right) from ADM rats or NP rats.	42
Figure 2-4	Composite drawings showing FLI and/or NADPH-d labeled neurons at three levels of the PVN in ADM rats or NP rats.	43
Figure 2-5	Numbers of neurons labeled for FLI in NTS, AP, and SFO from ADM rats or NP rats.	44
Figure 2-6	Photographs showing FLI labeled neurons in rats with intact AP or with AP ablation.	45
Figure 2-7	Changes in MAP after ADM injections in rats with AP ablation or AP sham ablation.	46
Figure 2-8	Numbers of neurons labeled for FLI and/or NADPH-d in three regions of the PVN (left), and four subdivisions of the middle PVN (right) after ADM injections in rats with AP ablation or AP sham ablation.	47
Figure 3-1	Photomicrographs showing the distribution of ppADM mRNA in the rat forebrain.	77
Figure 3-2	Photomicrographs showing the distribution of ppADM mRNA in the rat brainstem.	78
Figure 3-3	Photomicrographs showing the distribution of ppADM mRNA in the rat spinal cord and in the posterior pituitary.	79
Figure 3-4	Photomicrographs showing FLI labeled neurons and ppADM mRNA in the PVN, SON, and dorsal medulla through the AP, NTS and DMX, from control rats and LPS treated rats.	80
Figure 3-5	Photomicrographs showing ADM mRNA in the SFO from a control rat and a restrained rat.	81

Figure 3-6	Quantitative effects of LPS, restraint stress and dehydration on ppADM mRNA levels within autonomic centers including pPVN, mPVN, SON, ARC, Amy, VMH, NTS, DMX, AP, and SFO.	82
Figure 4-1	Changes in MAP in rats receiving one or two icv injections of ADM, or saline.	110
Figure 4-2	Changes in plasma ACTH levels in rats receiving one or two icv injections of ADM, or saline.	111
Figure 4-3	Nitrate/nitrite concentrations in hypothalamus and brainstem of rats receiving icv injections of ADM or saline.	112
Figure 4-4	Numbers of neurons with FLI in the subdivisions of the PVN, SON, ARC, NTS, AP, LC from rats receiving one or two icv injections of ADM, or saline.	113
Figure 4-5	Levels of CRF mRNA in the subdivisions of the pPVN, OXY and AVP mRNA in the mPVN, and TH mRNA in the LC, mNTS and dNTS in rats receiving one or two icv injections of ADM, or saline.	114

LIST OF ABBREVATIONS

ACTH, adrenocorticotrophic hormone

ADM, adrenomedullin

Amy, amygdala

AP, area postrema

AP-A, area postrema-ablated

AP-S, area postrema-sham surgery

AP, anterior lobe of the pituitary

ARC, arcuate nucleus

AVP, vasopressin

CA, catecholamine

CGRP, calcitonin gene-related peptide

CNS, central nervous system

CRF, corticotropin-releasing factor

CVO, circumventricular organ

DMX, dorsal motor nucleus of the vagus

dp, dorsal parvocellular division of PVN

FLI, Fos-like immunoreactivity

HPA, hypothalamo-pituitary-adrenal axis

icv, intracerebroventricular

IML, intermediolateral cell column of the spinal cord

IP, intermediate lobe of the pituitary

IR, immunoreactivity

ISH, in situ hybridization

iv, intravenous

LC, locus coeruleus

LPS, lipopolysaccharide

MAP, mean arterial pressure

mp_d, dorsolateral medial parvocellular PVN

mp_v, ventral medial parvocellular PVN

mPVN, magnocellular PVN

NA, noradrenaline

NADPH-d, nicotinamide adenine dinucleotide phosphate-diaphorase

NT, neurotensin

NO, nitric oxide

NP, sodium nitroprusside

NTS, nucleus of the solitary tract

OXY, oxytocin

pm, posterior magnocellular PVN

PP, posterior lobe of the pituitary

ppADM, preproadrenomedullin

pPVN, parvocellular PVN

PVN, hypothalamic paraventricular nucleus

SFO, subfornical organ

SON, hypothalamic supraoptic nucleus

TH, tyrosine hydroxylase

VLM, ventrolateral medulla

VMH, ventromedial nucleus of the hypothalamus

CHAPTER 1

INTRODUCTION

1.1 Overview

ADM, initially isolated from human pheochromocytoma, is a 52-amino acid peptide and belongs to the calcitonin gene related peptide (CGRP) family (Kitamura et al., 1993a). ADM is encoded by the gene for the ADM precursor, preproadrenomedullin (ppADM), which contains the sequences of both ADM and proadrenomedullin N-terminal 20 peptide (PAMP) (Kitamura et al., 1993b). Apart from the original detection in the pheochromocytoma and normal adrenal medulla, ADM has been found ubiquitously in peripheral tissues and the brain (Ichiki et al., 1994; Sakata et al., 1994; Ueta et al., 1995; Washimine et al., 1995).

Plasma ADM concentration is increased in a number of diseases, such as hypertension (Ishimitsu et al., 1994; Kohno et al., 1996a), heart failure (Jougasaki et al.,1995), and sepsis (Hirata et al., 1996; Nishio et al., 1997). Lipopolysaccharide (LPS), a component of bacterial cell walls, elevates ADM gene expression in both cultured smooth muscle cells (Sugo et al, 1995), and various tissues in rats (Shoji et al., 1995). ADM binds to its receptor, either ADM specific receptor (Eguchi et al., 1994; Kapas et al., 1995) or $CGRP_1$ receptor (Edwards et al., 1996), and then activates cAMP (Kitamura et al., 1993), ion channels (Lang et al., 1997; Sabates et al., 1997) or other second messengers (Feng et al., 1994; Miura et al., 1995).

ADM elicits multiple biological functions to regulate fluid and electrolyte balance, and cardiovascular homeostasis. In the periphery, it causes hypotension (Kitamura et al, 1993a), positive inotropism and chronotropism (Parkes and May, 1997), diuresis and natriuresis (Ebara et al, 1994), and inhibits aldosterone and ACTH release from the adrenal gland (Yamaguchi et al, 1995) and pituitary gland (Samson

et al, 1995), respectively. In the central nervous system, it induces hypertension (Takahashi et al., 1994), and inhibits drinking (Murphy and Samson, 1995), salt intake (Samson and Murphy, 1997), feeding (Taylor et al.,1996), and gastric emptying (Martinez et al., 1997). However, the role of ADM in the regulation of neuroendocrine and autonomic functions has not been well understood.

1.2 Adrenomedullin

1.2.1 Discovery and synthesis

In 1993, a Japanese group initially isolated a new peptide from human pheochromocytoma by its ability to elevate platelet cyclic adenosine 3',5'-monophosphate (cAMP) levels (Kitamura et al., 1993a). As this peptide was also abundant in normal adrenal medulla, it was termed "adrenomedullin (ADM)". Human ADM is a 52-amino acid peptide with a unique 6-amino acid residue ring structure formed by a disulfide and an amidated tyrosine at the carboxy terminus. These structures are essential for the receptor binding of ADM and for its biological activity. It shows 27% homology with calcitonin gene related peptide (CGRP), and therefore was thought to belong to the CGRP family (Kitamura et al., 1993a).

The gene for human ADM was cloned from human pheochromocytoma (Kitamura et al., 1993b). It encodes a 185-amino acid preproadrenomedullin. containing the sequences of both ADM and another peptide, proadrenomedullin N-terminal 20 peptide (PAMP). Following removal of the N-terminal signal peptide. the human preproadrenomedullin is processed to proadrenomedullin with 164-amino acids, and then by enzymatic cleavage, further processed to two biologically active

peptides: ADM and PAMP (Fig. 1). Among species the coding sequences and the level of peptide homology are highly conserved. (Sakata et al., 1993).

Although adrenomedullin has been shown to be stored in secretary granules in the pancreas (Martinez et al., 1996), little is known of its storage in other endocrine tissues. To date no evidence for intracellular storage of ADM is available. It has been shown that ADM immunoreactivity was detected in the culture medium of SW-13 cells, an adrenocortical adenocarcinoma cell line, but not in the extract of these cells (Takahashi et al., 1998), and that the synthesized ADM was not stored but constitutively secreted from endothelial cell and vascular smooth muscle cell (Isumi et al., 1998).

1.2.2 Distribution

Although originally identified in pheochromocytoma cells and normal adrenal medulla, ADM immunoreactivity (IR) have been found in various tissues, including adrenal cortex, kidney, lung, heart, spleen, small intestine, salivary gland, and brain of humans and rats (Ichiki et al., 1994; Sakata et al., 1994; Ueta et al., 1995; Washimine et al., 1995), as illustrated in Table 1. Likewise, ADM mRNA is also widely expressed in these tissues (Martinez et al., 1995; Miller et al., 1996; Nishimura et al., 1997). Whereas recent findings have shown that ADM peptide is distributed throughout the brain, with high levels in the pituitary gland, thalamus and hypothalamus (Satoh et al., 1995; Serrano et al., 2000), the detailed distribution of ADM gene expression in the CNS was unknown.

1.2.3 Regulation of production and secretion

The normal plasma concentration of ADM is in the range of 1 to 10 pg/ml. Its half life is 22 minutes and metabolic clearance is 27.4 ml/kg/min (Meeran et al., 1997). Plasma ADM is reported to be significantly increased in a number of human disease states, including hypertension (Ishimitsu et al., 1994; Kohno et al., 1996a), heart failure (Jougasaki et al.,1995), liver disorder (Cheung and Leung, 1997), renal failure (Ishimitsu et al., 1994; Cheung and Leung, 1997), acute asthma (Kohno et al., 1996b), and septic shock (Hirata et al., 1996; Nishio et al., 1997). However, whether elevated ADM is the cause or the consequence of these diseases remains unknown.

Studies of the in vivo regulation of ADM gene transcription and peptide synthesis have been carried out primarily in cultured vascular smooth muscle cells (VSMC) and endothelial cells (EC) where ADM mRNA is highly expressed. ADM synthesis from VSMC is augmented by various cytokines, growth factors, and hormones, including tumor necrosis factor, interleukin 1α and β, cortisol, aldosterone, and thyroid hormone (Sugo et al., 1994; Imai et al., 1995; Minamino et al, 1995). Lipopolysaccharide (LPS), bacterial cell wall lipopolysaccharide, increased ADM gene expression in rat aortic smooth muscle cells (Sugo et al, 1995) and in rat tissues, such as lung, adrenal gland, kidney, liver, skeletal muscle, ileum, jejunum (Shoji et al., 1995). Compared to the established factors known to control ADM gene expression in the periphery, relatively little is known of the regulation of ADM gene expression in the CNS.

1.2.4 Receptors and signal transduction

The observations that some actions of ADM were abrogated by $CGRP_{8-37}$, an antagonist of $CGRP_1$ receptor (Nuki et al., 1993; Entzeroth et al., 1995; Zimmermann et al., 1995; Mazzocchi et al., 1996; Taylor et al., 1996) suggest that at least some of the effects of ADM are mediated by $CGRP_1$ receptor which has a lower affinity to ADM than CGRP (Edwards et al., 1996). In addition, a specific ADM receptor has been reported to exist with a higher affinity to ADM than to CGRP (Eguchi et al., 1994), and this receptor has been cloned (Kapas et al., 1995).

Another orphan receptor called calcitonin receptor-like receptor (CRLR) has been cloned, and it is related to CGRP/ADM receptor (Njuki et al., 1993). Human CRLR, a 461-amino acid seven-transmembrane protein is expressed in the lung, heart, and kidney (Fluhmann et al., 1995). Subsequently, a receptor-activity modifying protein (RAMP-1) of 148 amino acids with a single transmembrane domain was cloned, and was shown to participate in transporting CRLR to the cell surface (McLatchie et al., 1998). Two further members of the RAMP family, RAMP-2 and RAMP-3, were also identified (McLatchie et al., 1998), and the current hypothesis is that RAMP2 and CRLP generate an ADM receptor, while RAMP1 and CRLP generate a $CGRP_1$ receptor (McLatchie et al., 1998) .

In addition to the originally identified signal pathway of cAMP (Kitamura et al., 1993), ADM receptors are also linked to ion channels including calcium and potassium channels (Lang et al., 1997; Sabates et al., 1997) and to a range of second messenger systems (Feng et al., 1994; Miura et al., 1995).

1.2.5 Biological functions

ADM exerts multiple effects in a variety of tissues coordinated to regulate fluid, electrolyte, and cardiovascular homeostasis. Biological effects of ADM are summarized in Table 1-2.

First described as a potent vasodilator (Kitamura et al, 1993a), ADM exerts this effect largely through the generation of NO in the vasculature (Gardiner et al, 1995). ADM has been reported to act directly on the heart to exert positive inotropism and chronotropism (Parkes and May, 1997). In the kidney, it elicits diuretic and natriuretic effects by increased renal blood flow and direct tubular actions (Ebara et al, 1994). In addition, it also inhibits aldosterone release from the adrenal gland (Yamaguchi et al, 1995) and ACTH secretion from the pituitary gland (Samson et al, 1995).

In the CNS, ADM suppresses water (Murphy and Samson, 1995) and salt intake (Samson and Murphy, 1997), effects which are complimentary to its renal diuresis and natriuresis. On the other hand, central administrations of ADM increase blood pressure and sympathetic activity (Takahashi et al., 1994), effects which are opposed to its peripheral vasodilation. Additionally, central ADM inhibits feeding (Taylor et al.,1996) and gastric emptying (Martinez et al., 1997). However, it is unknown how circulating ADM gains access to the brain to affect neuroendocrine and autonomic functions, nor is it clear how centrally administered ADM regulates neuroendocrine and autonomic functions.

1.3 *c-fos* acts as a marker of activated neurons

The genes responsive to external stimuli fall into two classes, immediate early genes, whose transcription is activated rapidly and transiently within minutes of stimulation, and late response genes, whose expression is induced more slowly over hours upon stimulation (Curran and Morgan, 1995). The expression of *c-fos* is induced by a variety of extracellular stimuli including polypeptide factors, ion channels, neurothasmitters, and excitatory aminoacids. Cell surface stimulation triggers second messenger signals that result in a transient elevation of *c-fos* transcription. Fos forms a heterodimeric transcription factor with the product of another immediate early gene, Jun, and binds to DNA sequences containing activator protein 1 (AP-1) sites to regulate the late response genes (Sheng and Greenberg, 1990; Curran and Morgan, 1995).

The expression of *c-fos* has been widely used as a marker of activated neurons in the brain (Krukoff, 1998). The basal expression of *c-fos* is very low in unstimulated neurons, but the transcription of *c-fos* occurs within minutes of application of stimulus (Herdegen et al., 1995; Krukoff et al., 1995; Krukoff and Khalili, 1997; Shan and Krukoff, 2000). Therefore, the presence of Fos within nuclei of neurons using immunohistochemistry is a common technique for identifying activated neurons (Krukoff, 1998). Its adventages are as following: (i) it is straightforward and easy to use; (ii) results can be quantitated; (iii) Fos immunohistochemistry can be combined with other anatomical techniques; and (iv) Fos expression illustrates multisynaptic pathways of the brain (Krukoff, 1998).

The use of Fos immunorhistochemistry also has limitations (Krukoff, 1998). (i) Careful controls are required to eliminate extraneous sources of background activity; (ii) Fos is not a universal marker of neuronal activity; (iii) Fos expression is only limited to excitatory pathways; (iv) Fos expression may not be suitable for long-term experiments; and (v) Fos expression does not distinguish whether an identified pathway is uni- or multisynaptic.

1.4 Involvement of nitric oxide

Nitric Oxide (NO) acts as a gaseous nonconventional neurotransmitter in the brain (Vincent, 1994; Krukoff, 1999). Three isoforms of NO synthase have been identified as being responsible for synthesizing NO (Forstermann et al., 1994; Forstermann et al., 1995). All isoforms use L-arginine and molecular oxygen as a substrate, and the final products are NO and citrulline. This process requires cofactors including NADPH, biopterin, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN) (Moncada et al., 1991).

Isoform I or neuronal NOS (nNOS) is constitutively expressed in central and peripheral neuronal cells, and in epithelial cells of lung, kidney, uterus, stomach, and kidney (Schmidt et al., 1992). nNOS is calcium-dependent and has been implicated in regulation of synaptic transmission in the CNS, blood pressure, smooth muscle relaxation, and vasodilation (Forstermann et al., 1994). Isoform II or inducible NOS (iNOS) is not expressed under basal conditions, but can be induced in many cell types including macrophages, endothelial cells, hepatocytes, and glia by lipopolysaccharide and cytokines (Bandaletova et al., 1993),. iNOS, the calcium-independent isoform,

participates in the pathophysiology of autoimmune diseases and septic shock (Forstermann et al., 1994). iNOS produces large amounts of NO that have cytotoxic effects, because NO can inhibit numbers of key enzymes (Nathan and Hibbs, Jr., 1991), and NO can directly interfere with the DNA of target cells and cause strand breaks and fragmentation (Wink et al., 1991). Isoform III or endothelial NOS (eNOS) has been found mostly in endothelial cells (Pollock et al., 1993). Like nNOS, eNOS is constitutively expressed and calcium-dependent (Forstermann et al., 1994; Forstermann et al., 1995). NO produced from endothelial cells dilates blood vessels, inhibits leukocyte adhesion to vascular endothelium, and inhibits prolification of vascular smooth muscle cells (Forstermann et al., 1994).

The NADPH-diaphorase histochemical reaction has been widely used to localize NO-producing neurons in the brain (Krukoff, 1998; Krukoff, 1999; Yang et al., 1999). The histochemical technique is based on the ability of NADPH-d to reduce soluble tetrazolim salts to a blue precipitate (Hope et al., 1991). In fixed tissues, NADPH-d staining has been shown to correlate with nNOS immunoreactivity (Hope et al., 1991; Dawson et al., 1991; Blottner et al., 1995).

1.5 Hypotheses

A large body of evidence from anatomical and functional observations suggests that ADM affects the activity of the autonomic nervous system. The three sets of experiments demonstrated in this thesis were designed to test the following hypotheses:

1. Circulating ADM can communicate with the brain through the CVO(s) to activate autonomic centers.
2. ADM mRNA is widely distributed in the rat central nervous system, and is affected by physiological manipulations.
3. Central ADM participates in regulating neuroendocrine and autonomic functions to maintain body homeostasis.

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Table 1-1: Distribution of ADM–like immunoreactivity in the human and rat.

Tissue/organ	Man	Rat
Adrenal medulla	+	+
Blood vessels	+	+
Kidney	+	+
Pituitary	+	+
Heart	+	+
Lung	+	+
Thyroid	+	+
Submandibular gland	-	+
Liver	+	+
Pancreas	+	+
Stomach	+	+
Intestine	+	+
Testis	-	+
Cerebral cortex	+	+
Cerebellum	+	+
Brainstem	+	+
Hypothalamus	+	+
Hippocampus	+	+

Table 1-2: Multiple biological actions of ADM gene-derived peptides

Tissue	Effects of ADM	Effects of PAMP
Vasculature	Vasodilation, antimitogenesis	Vasodilation
Heart	Positive chronotropism and inotropism, increased coronary blood flow, antimitogenesis	Unknown
Lung	Bronchodilation	Bronchodilation
Kidney	Natriuresis, diuresis, increased renal blood flow, stimulation of renin release	Unknown
Adrenal gland	Inhibition of aldosterone secretion	Inhibition of catecholamine and aldosterone release
Pituitary	Inhibition of ACTH secretion	Inhibition of ACTH release
Brain	Activation of the sympathetic nervous system (hypertension), inhibition of thirst, salt appetite, gastric emptying, inhibition of vasopressin release, increased collateralization and increased cerebral blood flow	Activation of the sympathetic nervous system (hypertension)

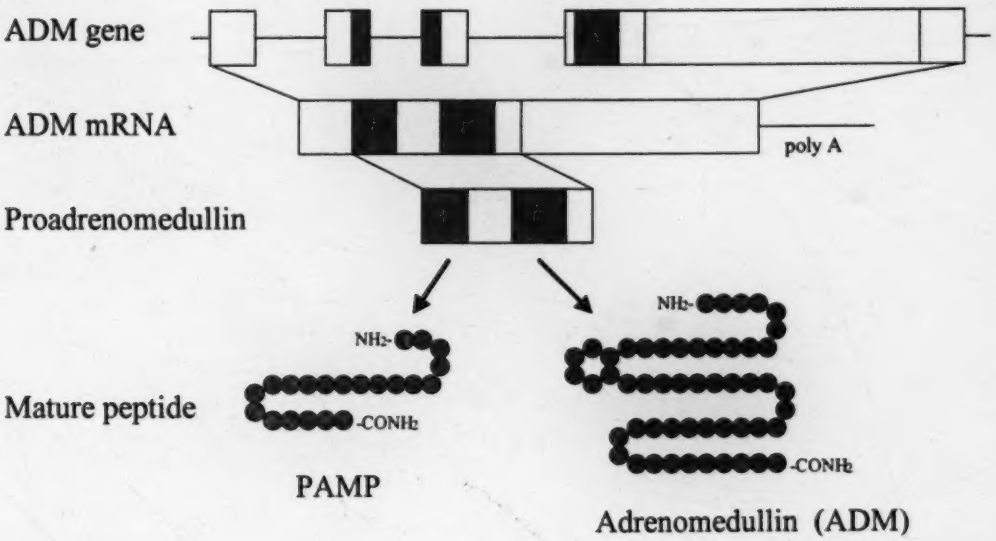

Figure 1-1: The ADM gene, precursor and biosynthesis of ADM and proadrenomedullin N-terminal 20 peptide (PAMP).

CHAPTER 2

Area Postrema Ablation Attenuates Activation of Neurons in the Paraventricular Nucleus in Response to Systemic Adrenomedullin

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2.1 ABSTRACT

Adrenomedullin (ADM) is a potent vasodilator in the periphery which also acts centrally to increase blood pressure and inhibit drinking, feeding and salt appetite. This study was designed to study the effects of circulating ADM on neuronal activation in autonomic centers in the rat brain and to examine whether neuronal NO may participate in these processes. We identified activated neurons 1 h after intravenous (iv) injections of ADM (2 nmol/kg) using immunohistochemistry for Fos. The nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemical reaction was used to localize putative NO-producing neurons and double labeling for Fos and NADPH-d was used to identify activated NO producing neurons. To separate baroreceptor-induced neuronal activation in autonomic centers by ADM from other effects it may have, iv infusions of sodium nitroprusside (NP) were used to mimic the hypotensive effects of ADM in control rats. Significantly greater numbers of activated neurons were found in the paraventricular nucleus of the hypothalamus (PVN) and especially in the dorsolateral medial parvocellular division, the nucleus of the solitary tract (NTS), and the area postrema (AP) of ADM-treated rats compared to control rats. In addition, the number of activated NO-producing neurons in the PVN was significantly higher in ADM-treated rats compared to rats treated with NP. To determine whether AP is one of the possible routes through which systemic ADM enters the brain to exert its central effects, the APs of rats were ablated by aspiration. One hour after iv injections of ADM, significantly fewer PVN neurons were activated in AP ablation rats compared to AP sham ablation rats. Similarly, the number of activated NO-producing neurons in the PVN was significantly lower in AP ablation rats compared to AP sham ablation rats. In conclusion,

our results suggest that systemic ADM gains access to the brain through the AP to regulate neuronal activity in autonomic centers and that neuronal NO might be involved in central autonomic and/or neuroendocrine regulation by ADM.

2.2 INTRODUCTION

Adrenomedullin (ADM), a novel 52 amino-acid peptide originally isolated from human pheochromocytoma tissue, is one of the most potent, peripherally hypotensive, neuropeptides known (Kitamura et al., 1993a). Immunoreactive ADM, ADM mRNA, and receptors recognizing ADM have recently been found not only in peripheral tissues but also in the brains of human and rat (Kitamura et al., 1993b; Ichiki et al., 1994; Owji et al., 1995; Oliver et al., 1998). Systemic administration of ADM causes vasodilation (Kitamura et al., 1993a), and diuretic and natriuretic actions in the kidney (Vari et al., 1996), whereas central administration induces hypertension (Saita et al., 1998) and reduces water (Murphy and Samson, 1995), salt (Samson and Murphy, 1997), and food (Taylor et al., 1996) intake.

The paraventricular nucleus of the hypothalamus (PVN) is an important autonomic center that integrates a wide variety of information including baroreceptor inputs and humoral inputs. Baroreceptor information is neurally relayed to the brain with the initial synapse found in the nucleus of the solitary tract (NTS) in the brainstem. This information is then transferred to other autonomic centers throughout the brain, including the PVN (Swanson and Sawchenko, 1983). Humoral inputs such as circulating peptides can gain access to the brain through circumventricular organs (CVOs) lacking a blood-brain barrier, which in turn, project directly or indirectly to the PVN to affect autonomic

functions (Leslie, 1986; Bishop and Hay, 1993; Ferguson and Bains, 1996). The effector neurons of the PVN comprise magnocellular and parvocellular neurons: magnocellular neurons affect the functions of the posterior pituitary, whereas parvocellular neurons influence sympathetic output or activity of the hypothalamic-pituitary-adrenal (HPA) axis (Swanson and Sawchenko, 1983).

The area postrema (AP), a CVO located on the dorsal surface of the medulla immediately adjacent to the NTS, has a rich vascular supply (Wislocki and Putman, 1920), and is thought to be a gateway for entry of circulating vasoactive peptides, such as angiotensin II (Ferguson and Bains, 1997), vasopressin (Hasser et al., 1997) and endothelin (Ferguson and Smith, 1991), into the brain. That ADM may gain access to the brain through the AP was suggested by *in vitro* extracellular single unit recording studies showing that ADM has direct excitatory effects on AP neurons (Allen and Ferguson, 1996), and *in vivo* studies showing that microinjections of ADM produce a transient increase in blood pressure and heart rate (Allen et al., 1997).

Nitric oxide (NO) acts as a gaseous, non-conventional neurotransmitter in the brain (Vincent, 1994), and has been proposed to be involved in the central regulation of autonomic functions by affecting sympathetic output to the periphery (Krukoff, 1999). Whether NO mediates the central regulation of autonomic functions by ADM, however, remains unclear.

The present study tested the hypothesis that circulating ADM gains access to the central nervous system through the AP to regulate autonomic functions. Using the expression of Fos, the protein product of the immediately-early gene c-*fos*, as a marker of activated neurons (Krukoff, 1998), we compared neuronal activation in the PVN, NTS,

and AP of ADM-treated rats and nitroprusside (NP)-treated rats. The NP-treated rats acted as controls to eliminate baroreceptor-induced neuronal activation in theses areas. Furthermore, we compared neuronal activation in the PVN of AP ablation rats and sham ablation rats to determine if destruction of the AP would attenuate the neuronal activation in the PVN in response to ADM. Finally, we used the nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemical reaction to localize putative NO-producing neurons (Krukoff and Khalili, 1997) in the above experiments to determine the proportions of NO neurons which were activated in the PVN.

2.3 MATERIALS AND METHODS

2.3.1 Animals.

Male Sprague-Dawley rats (250-300 g) were purchased from the Biological Animal Center, University of Alberta. They were housed in a 12-h:12-h light/dark cycle at 22°C and given free access to food and water. All protocols were approved by the local Animal Welfare Committee.

2.3.2 Experiment protocols.

2.3.2.1 ***Experiment 1.*** Rats were anaesthetized with sodium pentobarbital (50 mg/kg; i.p.; Somnotol, M.T.C. Pharmaceuticals, Hamilton, Canada) and the descending aorta and vena cava were cannulated and exteriorized as described previously (Krukoff et al., 1995). Wounds were sutured closed and rats were allowed to recover from anaesthesia. Rats were handled individually on a daily basis so that they became accustomed to these procedures. Four to 6 days after the initial surgery, the arterial line and venous line were flushed with heparinized saline (10 IU/ml). The arterial line was connected to a pressure

transducer to record arterial pressure and the rats were left undisturbed for 1 h before experimentation. The rats were divided into two groups. Rats in the first group received intravenous (iv) injections of adrenomedullin (ADM, 2 nmol/kg; #9508, Peninsula Laboratories, Inc; Belmont, CA, USA) dissolved in saline; rats in the second group received iv infusions of sodium nitroprusside (NP, 5.0 μg/kg/min for 10 min, #S-0501, Sigma Chemicals, St. Louis, MO, USA) using a Harvard infusion pump, and the rate of infusion was titrated to mimic the change in arterial pressure attained after ADM injections. The arterial pressures were monitored continuously for 1 h and rats were then deeply anaesthetized with Somnotol and prepared for perfusion as described below.

2.3.2.2 ***Experiment 2.*** Under the same anaesthesia as Experiment 1, rats were placed in a stereotaxic instrument with the head ventroflexed. After the skin and muscles were dissected along the midline, the cisterna magna was opened to expose the dorsal surface of the medulla. The area postrema (AP) was visualized with an operating microscope and aspirated through a 30-gauge blunted stainless steel tube connected to a vacuum line as described previously (Wang and Edwards, 1997; Lee et al., 1998). Sham lesion surgeries of rats were identical except that no vacuum was applied. The muscles and skin were sutured and rats were allowed to recover from anaesthesia. Four to six days after the initial surgery, rats were weighed, deeply anaesthetized, and prepared for perfusion as described below.

2.3.2.3 ***Experiment 3.*** Under the same anaesthesia as Experiment 1, rats were subjected to AP ablation (AP-A) or sham surgery (AP-S) with arterial and venous catheterization in the same surgical session. Four to six days after the surgery, the arterial pressures of rats were recorded through the arterial lines and the rats were left undisturbed for 1 h before

receiving iv injections of ADM through the venous lines as described in Experiment 1. One hour after ADM injections, rats were weighed, deeply anaesthetized, and prepared for perfusion as described below.

2.3.3 Processing of tissues.

Rats were perfused transcardially with 200 ml saline followed by 500 ml ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The brains were removed, postfixed in half-strength fixative and 10% sucrose for 1 h, and stored in 20% sucrose overnight at 4°C. Coronal sections (50 μm) of the brain were cut in a cryostat, and sections of brainstem and forebrain were incubated overnight at room temperature with rabbit anti-c-*fos* antiserum (#PC38, Oncogene, Uniondale, NY, USA) diluted 1:4000 in 0.3% Triton X-100/phosphate-buffered saline (PBS, pH 7.2). According to the supplier's specifications, this antibody recognizes Fos and Fos-related protein; therefore, the staining we have obtained will be described as Fos-like immunoreactivity (FLI). The following day, tissues were processed using the avidin-biotin immunoperoxidase method (ABC Vecta Stain Kit; Vector laboratories, Burlingame, CA, USA). FLI was visualized as a brown reaction product with the chromagen diaminobenzidine (#D-5637, Sigma). After a rinse in PBS, sections were incubated for 20 minutes at 45°C in a solution containing 1 mg/ml β-NADPH (#N-1630, Sigma), 0.3 mg/ml nitroblue tetrazolium (#N-6876, Sigma) dissolved in dimethyl sulfoxide (#D-8779, Sigma), and 0.6% Triton X-100/PBS. Sections were mounted onto glass microscope slides, coverslipped with Elvanol (Moviol, Calbiochem Corporation, La Jolla, CA, USA; dissolved in 40 ml PBS and 20 ml glycerol), and examined with a Zeiss light microscope.

2.3.4 Subdivision of nuclei.

To aid in analysis, sections of the NTS were divided into caudal, middle, and rostral regions relative to the location of the AP. Caudal sections were between –14.6 and –14.0 mm caudal to bregma; middle, between –14.0 and –13.5 mm; and rostral, between –13.5 and –13.1 mm (Paxions and Watson, 1986; Krukoff et al., 1999). Sections from PVN were also divided into caudal, middle, and rostral regions. Caudal sections were between –2.00 and –1.85 mm caudal to bregma; middle, between –1.85 and –1.70 mm; and rostral, between –1.70 and –1.55 mm (Krukoff et al., 1997). In addition, the middle PVN was subdivided into the following functional divisions: posterior magnocellular (pm), dorsal parvocellular (dp), ventral medial parvocelluar (mp_v), and dorsolateral medial parvocellular (mp_d) divisions (Swanson and Sawchenko, 1983).

2.3.5 Analysis.

Only neurons with visible nuclei were counted. Neurons with FLI were counted in the PVN, NTS, AP or subfornical organ (SFO). Neurons with NADPH-d or FLI/NADPH-d (double labeled) were counted only in the PVN. For each subdivision considered, 3 to 5 sections were analyzed per rat. Counts were expressed as given values *per section* to eliminate factors related to double counting of neurons. In all analyses of mean arterial pressure and labeled neurons, n=4 for each group of rats. All data are expressed as means ± SE, and a Student's t-test was used to test for statistical significance between two groups ($P<0.05$).

2.4 RESULTS

2.4.1 *Experiment 1:* Activation of neurons in autonomic centers in response to iv administration of ADM or NP.

2.4.1.1 Mean arterial pressure

Figure 1 shows the change in mean arterial pressure in response to iv injections of ADM and iv infusions of NP. Mean arterial pressure was decreased by approximately 20 mmHg within the first 5 min of the experiment and gradually recovered within 30 min in both treatments.

2.4.1.2 Neuronal labeling in the PVN (Fig. 2A, B)

FLI labeled neurons: There were significantly higher numbers of neurons with FLI in the rostral, middle and caudal regions of the PVN, and in the dorsolateral medial parvocellular (mp_d) division of the middle PVN in ADM-treated rats compared to NP-treated rats (Fig. 3A).

NADPH-d labeled neurons: Whereas no significant differences in numbers of NADPH-d labeled neurons were found in the rostral, middle or caudal region of the PVN between ADM- and NP-treated rats, higher numbers of NADPH-d labeled neurons were found in the dorsal parvocellular (dp) division of the middle PVN in the ADM group compared to the NP group (Fig. 3B).

Double labeled neurons: Significantly higher numbers of double labeled neurons were found in the rostral, middle and caudal regions of the PVN, and in the dorsal parvocellular (dp), ventral medial parvocellular (mp_v) and dorsolateral medial parvocellular (mp_d) divisions of the middle PVN in ADM-treated rats compared to NP-

treated rats (Fig. 3C). Figure 4 summarizes the regional and divisional distribution of single-labeled and double-labeled neurons in the PVN in rats treated with ADM or NP.

2.1.4.3 Neurons with FLI in the NTS and CVOs (Fig. 2C, D)

Significantly higher numbers of neurons with FLI were found in all regions of the NTS in ADM-treated rats compared to NP-treated rats (Fig. 5A). Numbers of neurons with FLI were significantly higher in the AP in ADM-treated rats compared to NP-treated rats whereas no significant difference was found in the SFO (Fig. 5B).

2.4.2 *Experiment 2:* Activation of PVN neurons in AP-A rats or AP-S rats

This control experiment was done to determine if the AP ablation itself leads to induction of FLI in the PVN. Only rats with complete lesions of the AP and minimal damage to the adjacent NTS, confirmed in coronal brainstem sections with light microscopy, were analyzed (Fig. 6). No significant difference in numbers of neurons with FLI was found in PVN between AP-A rats (20.7 ± 2.4 per section) and AP-S rats (18.0 ± 1.0 per section). In addition, significant decreases in body weight were found 4 to 6 days after surgery in rats with AP-A (-10.1 ± 0.8 %) compared to rats with AP-S (-1.5 ± 1.1 %).

2.4.3 *Experiment 3:* Activation of PVN neurons in AP-A rats or AP-S rats in response to iv administration of ADM

2.4.3.1 Mean arterial pressure

The changes in mean arterial pressure in response to ADM were similar in AP-A and AP-S rats (Fig. 7). There was a transient decrease of about 20 mmHg in blood

pressure after ADM administration and blood pressure gradually returned to the base line level after about 30 min (Fig. 7).

2.4.3.2 Neuronal labeling in the PVN

FLI labeled neurons: Significantly lower numbers of neurons with FLI were found in the rostral, middle and caudal regions of the PVN, and in the dorsolateral medial parvocellular (mp_d) division of the middle PVN in AP-A rats compared to AP-S rats (Fig. 8A).

NADPH-d labeled neurons: No significant differences in NADPH-d labeled neurons were found in the rostral, middle or caudal region of the PVN between groups of rats; significantly lower numbers of NADPH-d labeled neurons were found in the dorsal parvocellular (dp) division of the middle PVN in AP-A rats compared to AP-S rats (Fig. 8B).

Double labeled neurons: Significantly lower numbers of double labeled neurons were found in all regions of the PVN and in all divisions of the middle PVN in AP-A rats compared to AP-S rats (Fig. 8C).

2.5 DISCUSSION

This study was designed to investigate the effects of circulating ADM on activity of neurons in a group of autonomic centers in the brain and to determine the possible route through which ADM may mediate these effects. The results illustrate that circulating ADM activates neurons in the PVN, NTS, and AP through a pathway which is not related to the baroreflex. Furthermore, removal of the AP attenuates the neuronal activation, providing strong evidence that ADM gains access to the brain through this

CVO to affect neuronal activity in the PVN and NTS. Finally, increased activation of NO-producing neurons in functionally identified divisions of the PVN in ADM treated rats suggests that NO may participate in the regulation of autonomic and/or neuroendocrine functions by circulating ADM.

It has been shown that stimulation of the baroreceptor reflex by hypotension induces Fos expression in the neuronal circuits between the NTS of the brainstem and the PVN of the hypothalamus (Krukoff et al, 1995, 1997). In order to separate baroreceptor-induced activation of autonomic neurons by ADM from other effects it may have, we compared the results from rats treated with ADM to those from rats whose arterial pressures were decreased to a similar degree using the peripheral vasodilator, NP. We found significantly higher numbers of activated neurons in the PVN and brainstem of ADM treated rats. Thus, while activation of some of these neurons is likely due to the peripheral depressor effects of ADM which activate baroreceptor reflex pathways, the increased numbers of activated neurons in ADM treated rats compared to NP treated rats provide evidence for activation of other pathways as well.

It has been reported that ADM inhibits basal and corticotropin-releasing hormone-stimulated ACTH secretion from cultured pituitary cells (Samson et al.,1995), and iv ADM infusion lowers circulating ACTH and cortisol levels in conscious sheep (Parkes and May, 1995). These studies suggest that ADM acts directly on the pituitary to suppress the activity of the adrenal gland (Parkes and May, 1995). Our results show that a significantly higher number of activated neurons was found in the dorsolateral medial parvocellular division (mp_d) of the middle PVN in ADM treated rats compared to NP treated rats. As these neurons project to the median eminence (Swanson and Sawchenko,

1983) to affect functions of the anterior pituitary, our results suggest that systemic ADM can affect the HPA axis, not only directly as suggested above, but also through hypothalamic neuronal pathways. We also show that the numbers of activated neurons found in the dorsal parvocellular (dp) and ventral medial parvocelluar (mp_v) divisions of the PVN were similar in ADM and NP treated rats. Because the neurons in both of these divisions project to other autonomic centers in the brain and spinal cord (Swanson and Sawchenko, 1983), the results suggest that activation of these neurons is related to stimulation of the baroreceptor reflex.

The next question we addressed is how systemic ADM can gain access to the central nervous system (CNS) to exert its effects. The AP and SFO are two important CVOs through which many vasoactive peptides, such as angiotensin II (Ferguson and Bains, 1997), vasopressin (Hasser et al., 1997), and endothelin (Ferguson and Smith, 1991) affect neuronal activity in the brain. The AP is adjacent to the NTS and has extensive projections to this brainstem area, while the SFO is more closely related to forebrain structures, both anatomically and functionally (Bishop and Hay, 1993; Ferguson and Bains, 1996). In our study, a significantly higher number of activated neurons was found in the AP, but not the SFO, in response to ADM administration compared to NP treatment, suggesting that systemic ADM gains access to the CNS, at least partly, at the AP. In addition, more activated neurons were found in the NTS of ADM treated rats compared to NP treated rats, suggesting that the signal from the AP was transferred through the NTS to reach the PVN. Moreover, while we cannot rule out the possibility that other CVOs are also involved in the signaling of ADM to the brain, removal of the AP significantly attenuated neuronal activation in the PVN, further

supporting our hypothesis that ADM gains access to the brain through the AP to regulate autonomic functions. The physiological relevance of this pathway as a route for ADM is supported by the finding that neurons in the AP are electrophysiologically responsive to ADM (Allen and Ferguson, 1996).

Interestingly, the central effects of ADM are opposite to its peripheral hypotensive effects. Both microinjection of ADM into the AP (Allen et al., 1997) and intracerebroventricular injection of ADM cause increases in arterial pressure (Saita et al., 1998). Therefore, it has been suggested that the central effects of ADM counteract the peripheral hypotensive effects to maintain homeostatic balance in the animal (Samson, 1998, 1999). Our results and those of others (Allen et al., 1997) suggest that the AP may be one of the routes through which circulating ADM acts centrally to mediate this homeostasis.

With the AP ablation experiment, we have ruled out the possibility that AP ablation itself can induce Fos expression in the PVN four to six days after surgery. We found that, although the ablation does not affect Fos expression, it leads to weight loss, consistent with previous studies which showed that AP ablation is associated with decreased food intake (Wang and Edwards, 1997). Interestingly, intracerebroventricular administration of ADM also inhibits feeding (Taylor et al., 1996). Together these studies suggest that ADM may affect ingestive behavior through its actions at the AP.

NO acts as a neurotransmitter which is hypothesized to participate in central homeostatic mechanisms (Krukoff, 1999). To determine if autonomic regulation by central ADM may also be mediated by NO, we compared the numbers of activated NO-producing neurons in the PVN of ADM treated rats with those from NP treated rats.

Although few differences in total numbers of NADPH-d labeled neurons were found in any region of the PVN between ADM and NP treatment, significantly greater numbers of double labeled neurons were found in all regions of the PVN in ADM treated rats. The activation of greater numbers of NO-producing neurons suggests, therefore, that NO is involved in autonomic and/or neuroendocrine regulation in response to systemic ADM, although the mechanism through which ADM may affect the neuronal NO system remains to be clarified.

In conclusion, by using Fos expression as a marker of activated neurons, we have shown that, apart from baroreflex neuronal activation due to its peripheral hypotensive effects, systemic ADM activates neurons in the PVN, NTS and AP. Furthermore, ablation of the AP attenuates this effect, confirming our hypothesis that systemic ADM gains access to the brain at the AP to affect neuronal function. In addition, the activation of neurons in the dorsolateral medial parvocelluar division of the middle PVN raises the possibility that ADM affects HPA axis activity through hypothalamic neuronal pathways. Finally, by combining NADPH-d staining with Fos immunohistochemistry to identify activated NO-producing neurons, our results lead us to propose that central regulation of autonomic and/or neuroendocrine functions by ADM may be at least partly mediated by NO.

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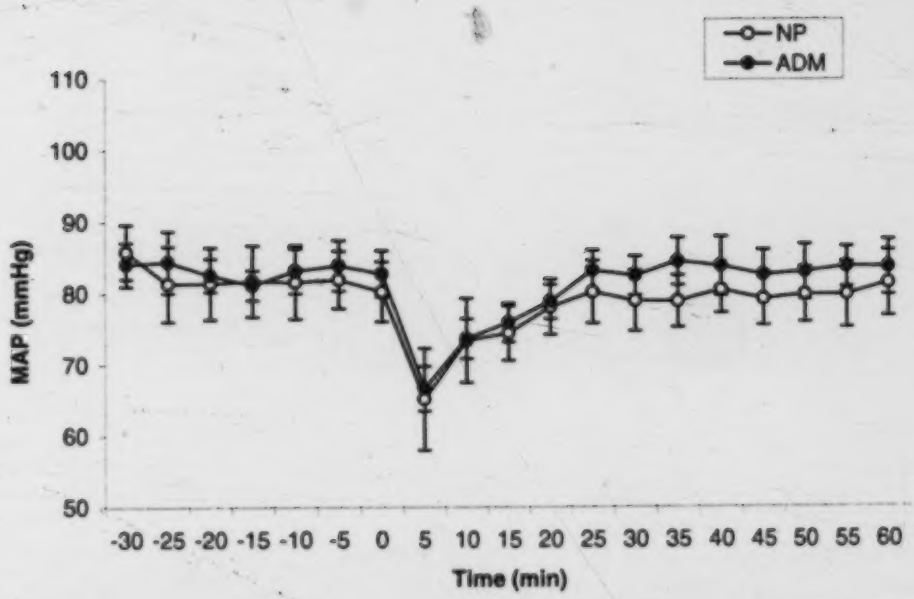

Figure 2-1: Changes in mean arterial pressure (MAP) in rats after single intravenous (iv) injections of adrenomedullin (ADM, 2 nmol/kg) or iv infusions of nitroprusside (NP, 5.0 μg/kg/min for 10 min) at 0 min. Values are expressed as mean ± SE (n=4 for each group of rats).

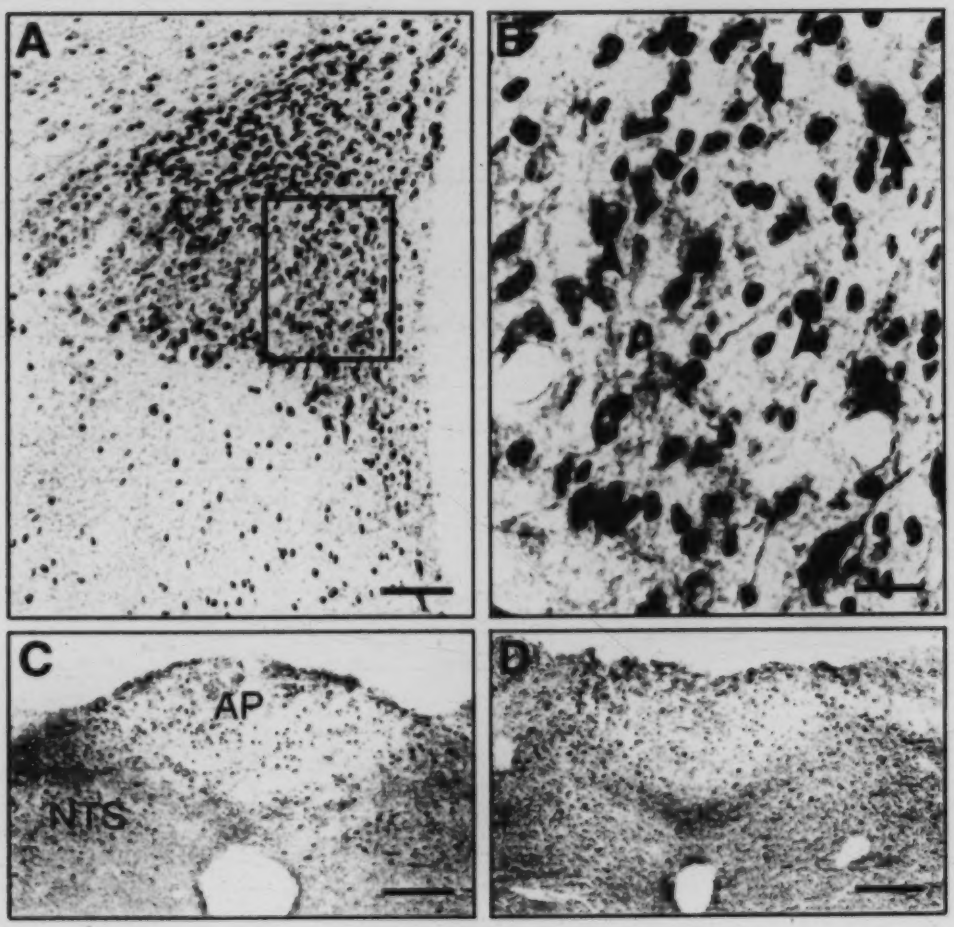

Figure 2-2: A, B: Fos-like immunoreactivity (FLI) and NADPH-d staining in the paraventricular nucleus of the hypothalamus of rats treated with adrenomedullin (ADM, 2 nmol/kg). The area within the rectangle in A is shown at higher magnification in B to show the single-labeled neurons with FLI or NADPH-d staining (arrowheads) and neurons double-labeled with FLI and NADPH-d (arrow). C, D: FLI in the area postrema (AP) and nucleus of the tractus solitarius (NTS) of rats treated with ADM (2 nmol/kg) (C) or nitroprusside (NP) (D). Scale bars in A, C, and D =100 μm; in B = 40 μm. For color images of similar types of labeling, see ref. 16.

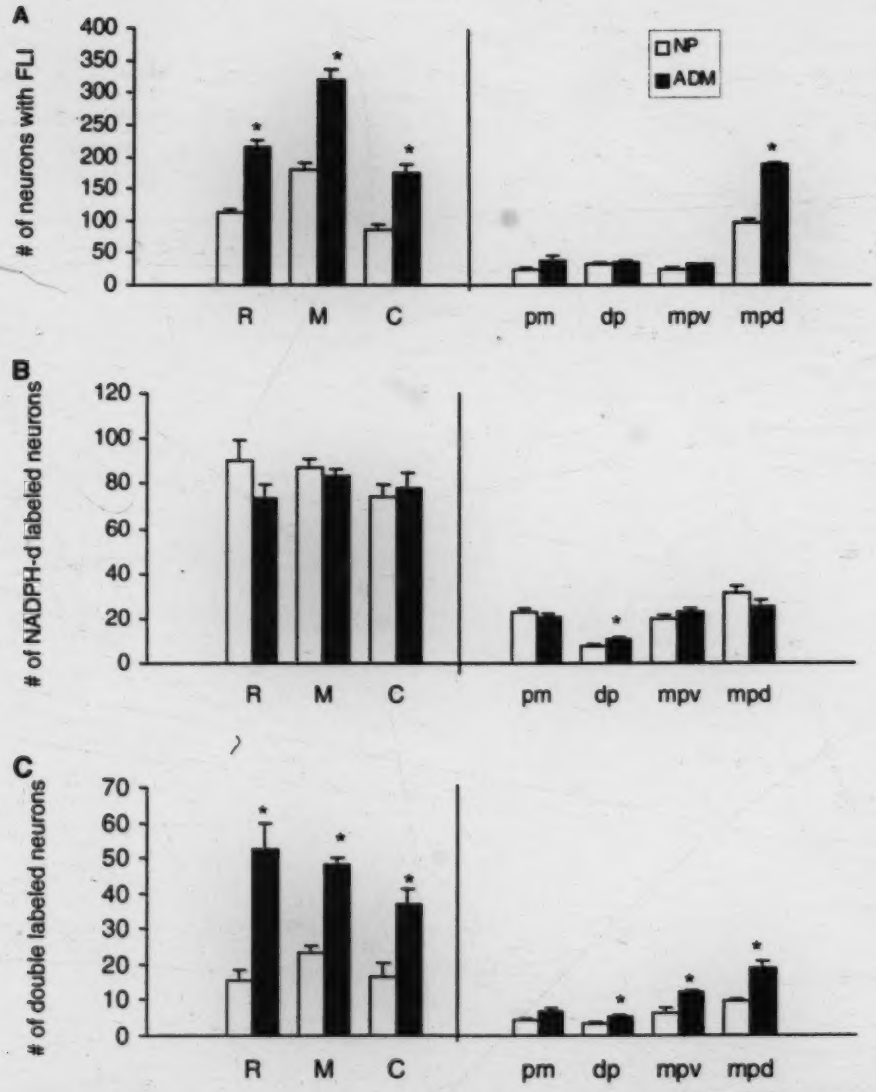

Figure 2-3: Neurons with FLI (A), NADPH-d staining (B), or double staining (C) in rostral (R), middle (M) and caudal (C) regions of the paraventricular nucleus (PVN) of the hypothalamus (left), and in the posterior magnocellular (pm), dorsal parvocellular (dp), ventral medial parvocelluar (mp_v), and dorsolateral medial parvocellular (mp_d) divisions of the middle PVN (right) from rats treated with iv injections of adrenomedullin (ADM) or iv infusions of nitroprusside (NP). Values are expressed as mean ± SE (n=4 for each group of rats); *, significant difference between groups ($P<0.05$).

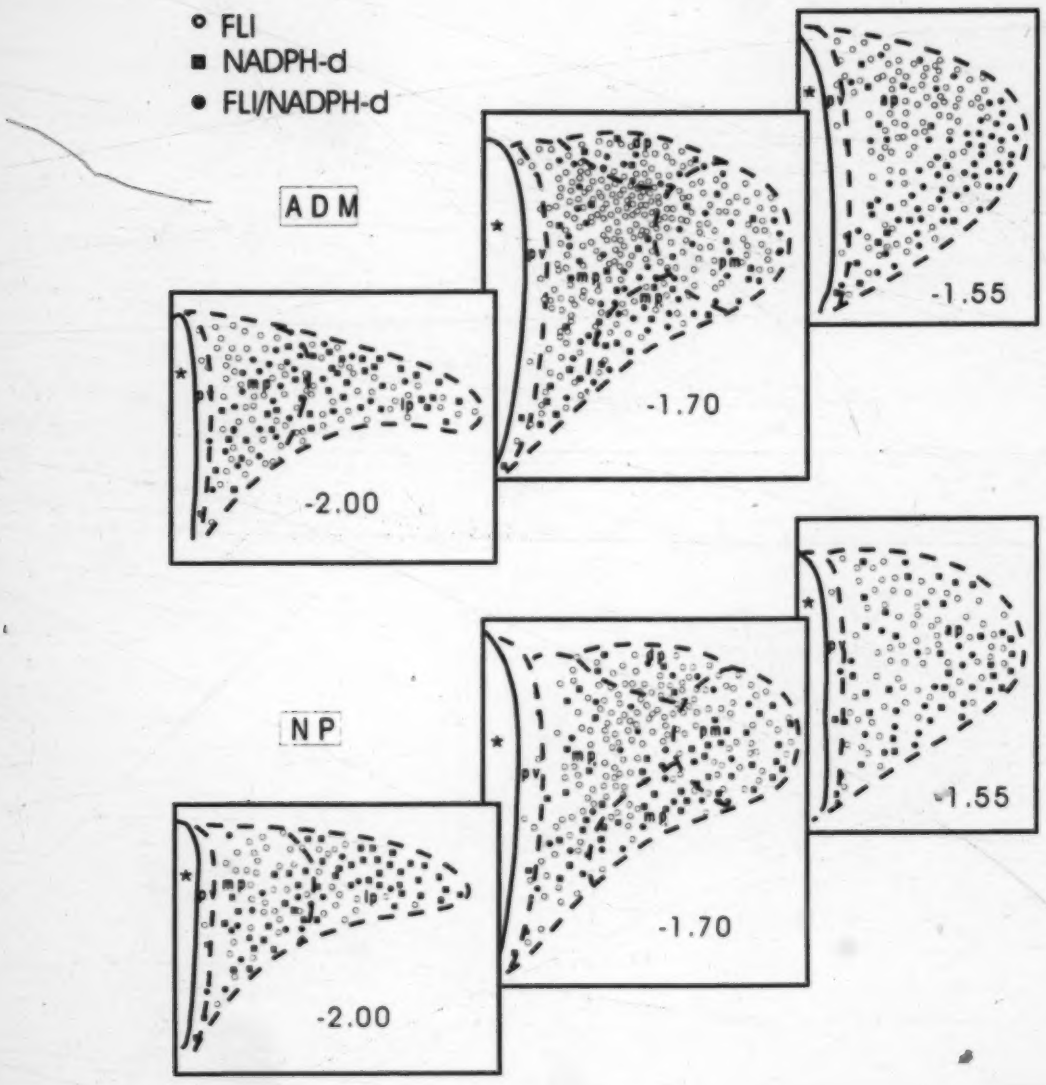

Figure 2-4: Composite drawings showing single- and double-labeled neurons at three levels of the paraventricular nucleus of the hypothalamus (PVN) in rats treated with adrenomedullin (ADM) (top) or nitroprusside (NP) (bottom). Numbers show rostrocaudal coordinates relative to Bregma (Paxinos and Watson, 1986). FLI, Fos-like immunoreactivity; NADPH-d, nicotinamide adenine dinucleotide phosphate-diaphorase; ap, lp, mp and dp, anterior, lateral, medial and dorsal parvocellular divisions of the PVN, pm, posterior magnocellular PVN, mp_v, ventral medial parvocellular PVN, mp_d, dorsolateral medial parvocellular PVN, pv, periventricular PVN. *, third ventricle.

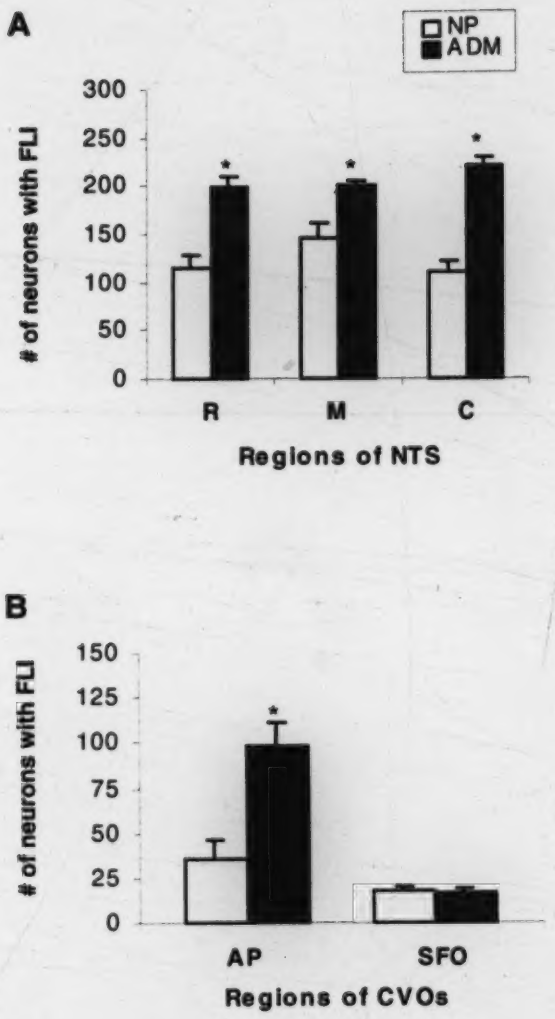

Figure 2-5: Neurons with Fos-like immunoreactivity (FLI) in rostral (R), middle (M) and caudal (C) regions of nucleus of the tractus solitarius (NTS) (A), and in the area postrema (AP) and subfornical organ (SFO) (B), from rats treated with adrenomedullin (ADM) or nitroprusside (NP). Va'ues are expressed as mean ± SE (n=4 for each group of rats); *, significant difference between groups ($P<0.05$).

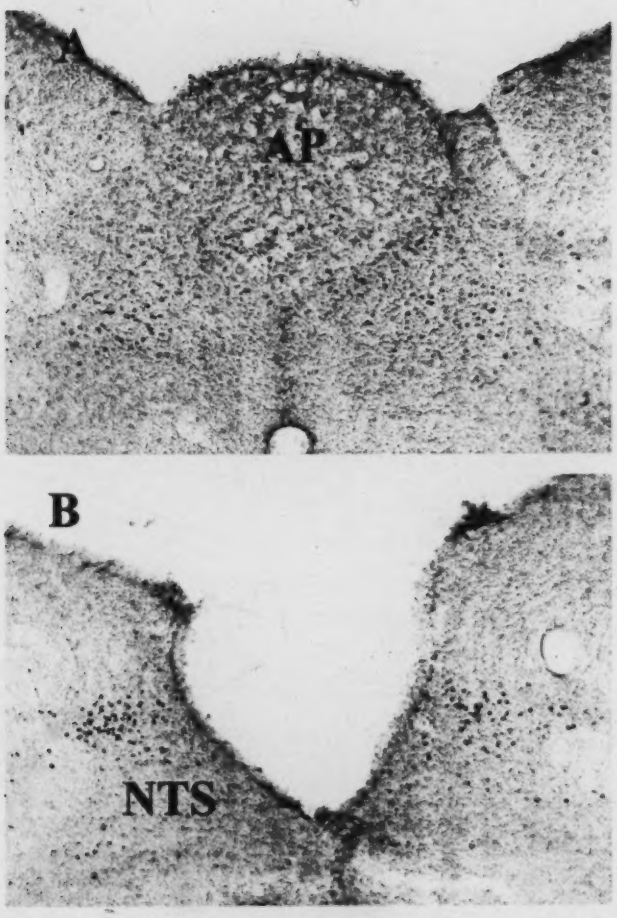

Figure 2-6: Photographs of coronal brainstem sections stained for Fos-like immunoreactivity (FLI) in rats with intact area postrema (AP) (A), or with AP ablation (B). NTS, nucleus of the tractus solitarius. Scale bars: 200 μm.

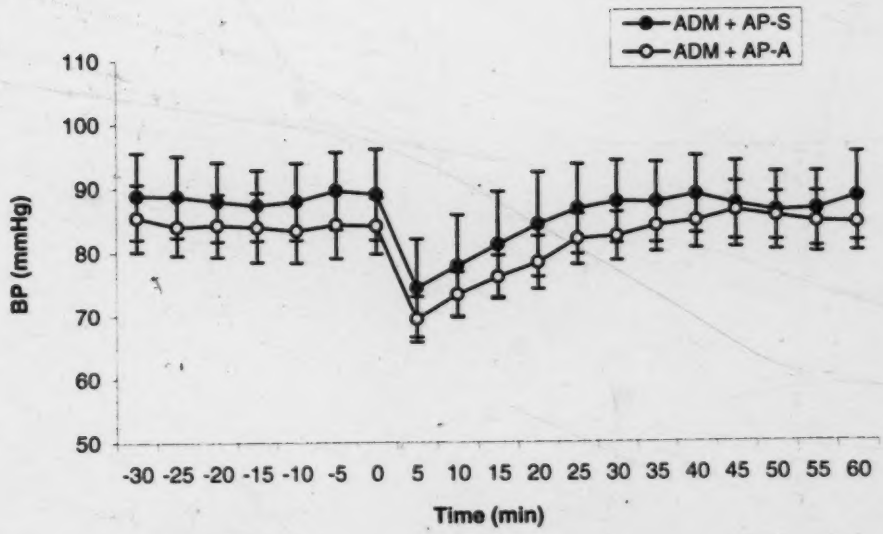

Figure 2-7: Changes in mean arterial pressure after intravenous (iv) injections of adrenomedullin (ADM) at 0 min in rats with AP ablation (AP-A) or AP sham ablation (AP-S). Values are expressed as mean ± SE (n=4 for each group of rats); *, significant difference between groups ($P<0.05$).

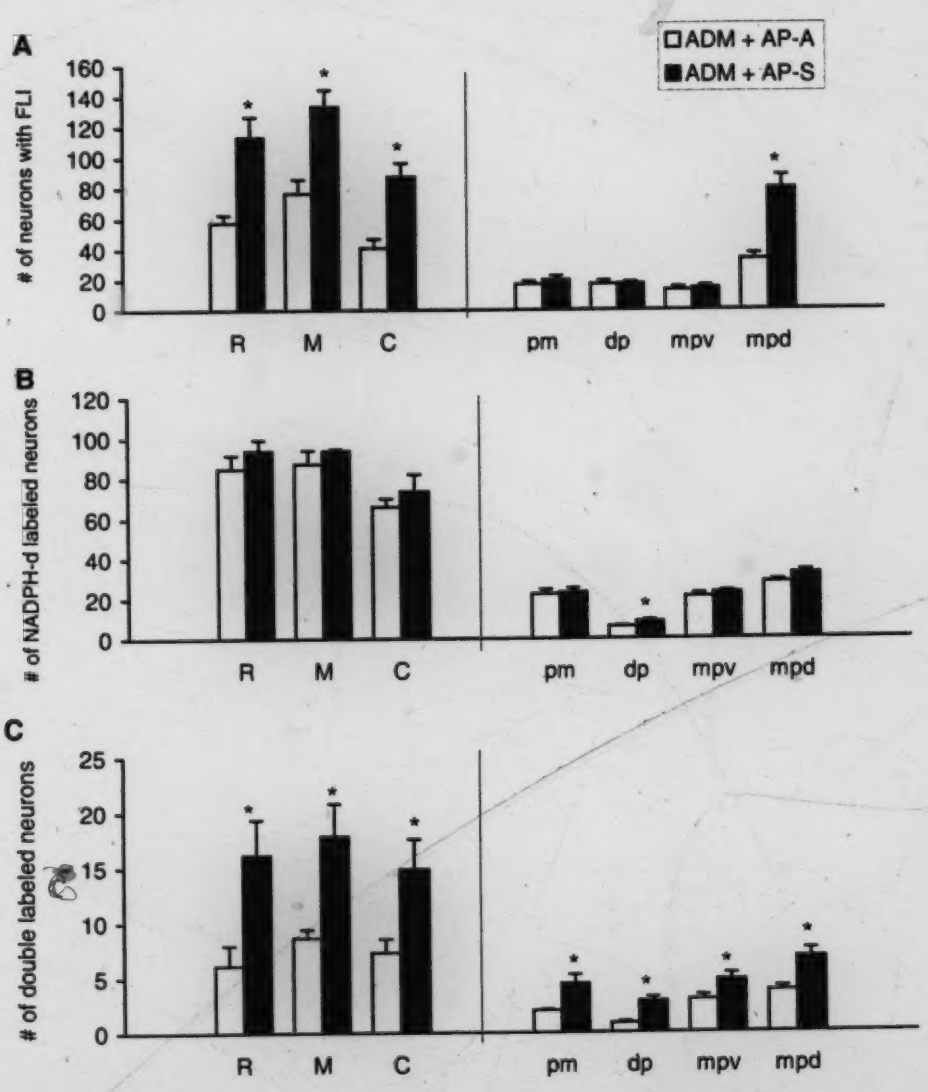

Figure 2-8: Neurons with Fos-like immunoreactivity (FLI) (A), NADPH-d staining (B), or double staining (C) in rostral (R), middle (M) and caudal (C) regions of the paraventricular nucleus (PVN) of the hypothalamus (left), and in the posterior magnocellular (pm), dorsal parvocellular (dp), ventral medial parvocellular (mp_v), and dorsolateral medial parvocellular (mp_d) divisions of the middle PVN (right) after iv injections of adrenomedullin (ADM) in rats with AP ablation (AP-A) or AP sham ablation (AP-S). Values are expressed as mean ± SE (n=4 for each group of rats); *, significant difference between groups ($P<0.05$).

CHAPTER 3

The Distribution of Preproadrenomedullin mRNA in the Rat Central Nervous System and Its Modulation by Physiological Stressors

(A version of this chapter is being revised for resubmission to the J. Comp. Neurol., and is used with permission from my supervisor Dr. T.L. Krukoff.)

3.1 ABSTRACT

Adrenomedullin (ADM), encoded by the preproadrenomedullin (ppADM) gene, exerts multiple functions in a wide variety of peripheral and central tissues. Although ADM-like immunoreactivity (IR) has been shown to be widely distributed throughout the rat central nervous system (CNS), the detailed distribution of ppADM gene expression in the CNS and its modulation by physiological stimuli remain unknown. In our study, in situ hybridization (ISH) was used to localize ppADM mRNA in the rat brain and to quantify its levels after exposure to different stressors including lipopolysaccharide (LPS), restraint stress, and dehydration. In addition, Fos immunoreactivity was used to identify the activation of neurons in response to LPS. Our results show that like ADM-IR, ppADM mRNA is widely distributed throughout the rat CNS, with especially high levels in autonomic centers such as hypothalamic paraventricular nucleus (PVN), hypothalamic supraoptic nucleus (SON), locus coeruleus (LC), ventrolateral medulla (VLM), and intermediolateral cell column of the spinal cord (IML). Furthermore, LPS (100μg/kg) inhibits ppADM gene expression in the parvocellular PVN (pPVN), magnocellular PVN (mPVN), SON, dorsal motor nucleus of the vagus (DMX), and area postrema (AP) among examined regions; restraint stress reduces ppADM mRNA levels in the pPVN, mPVN, SON, nucleus of the solitary tract (NTS), DMX, AP, and subfornical organ (SFO); 24 hours of water deprivation decreases ppADM gene expression only in the mPVN and SON. Based on these results, we hypothesize that physiological stressors lead to attenuation of ADM production in autonomic centers as the animal attempts to re-establish homeostatic balance in these systems.

3.2 INTRODUCTION

Adrenomedullin (ADM), encoded by the preproadrenomedullin (ppADM) gene, is a novel 52 amino-acid peptide. ADM is produced in cells of many tissues and organs (Ichiki et al., 1994; Sakata et al., 1994), and elicits potent vasodilatory effects (Kitamura et al., 1993a), diuresis, and natriuresis (Vari et al., 1996) when administered peripherally. On the other hand, central administration of ADM induces hypertension (Takahashi et al., 1994), and inhibits water (Murphy and Samson, 1995), salt (Samson and Murphy, 1997), and food intake (Taylor et al., 1996). In addition, ADM concentration is increased in the plasma and tissues under pathological conditions such as heart, hypertensive and renal diseases (Eto et al., 1999). Therefore, ADM has been proposed to be involved in regulation of the circulation and of body fluid balance.

The first reports showing the presence of ADM immunoreactivity (IR) (Ichiki et al.,1994; Sakata et al., 1994), ADM mRNA (Kitamura et al., 1993b) and ADM binding sites (Owji et al., 1995) in peripheral tissues such as lung, heart, spleen and adrenal gland, led investigators to focus on actions of ADM on these peripheral targets (Samson, 1998). To establish the physiological relevance of ADM, the regulation of ppADM gene expression in peripheral tissues by physiological manipulations has been documented. For example, intravenous (iv) administration of lipopolysaccharide (LPS), a bacterial endotoxin that activates the host immune response, markedly elevates plasma ADM and ppADM mRNA levels in the lung, heart, liver, ileum, and aorta (Shoji et al., 1995). In addition, restraint stress also increases ADM levels in the plasma, adrenal gland and pituitary (Khan et al., 1999).

Compared to the more established roles of ADM in the periphery, relatively little is known of the physiological relevance of central ADM. It has been shown that ADM elements are found in the pituitary gland and many regions of the brain including cerebral cortex, cerebellum, brainstem and hypothalamus (Hwang and Tang, 1999). In addition, in limited studies of the hypothalamus, ADM immunoreactivity (IR) has been localized to the hypothalamic paraventricular nucleus (PVN), supraoptic nucleus (SON; Ueta et al., 1995) and hypothalamo-neurohypophysial tract (Ueta et al., 1999). Whereas ADM IR has been recently found to be widely distributed throughout the central nervous system (CNS; Serrano et al., 2000), the detailed mapping of ADM gene expression in the CNS was lacking, and it was not known how physiological stimuli affect central ppADM gene expression. In the present study, in situ hybridization (ISH) was used to localize ppADM mRNA in the CNS and to measure changes in ppADM mRNA levels in response to various physiological stressors: LPS (an immune stressor), restraint stress, and dehydration. In addition, Fos immunohistochemistry is used to identify the neuronal activation following LPS.

3.3 MATERIALS AND METHODS

3.3.1 Animals

Male Sprague-Dawley rats (250-300 g) were purchased from the Biological Animal Center, University of Alberta. They were housed in a 12:12 hour light/dark cycle at 22°C and given free access to food and water. All protocols used in these experiments were approved by the local Animal Welfare Committee.

3.3.2 Experimental protocol

3.3.2.1 ADM mRNA survey

Three rats were anesthetized with sodium pentobarbital (50 mg/kg; i.p.; Somnotol, M.T.C. Pharmaceuticals, Hamilton, Canada) and perfused transcardially with 200 ml saline followed by 500 ml ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The brains were removed, postfixed in half-strength fixative and 15% sucrose for 1 hour, and stored in 20% sucrose overnight at 4°C. Coronal sections (40 μm) of the brainstem, forebrain, spinal cord, and pituitary were cut in a cryostat, and frozen sections were thaw mounted onto Superfrost®/Plus slides (Fisher Scientific) and stored at -70°C for later in situ hybridization (ISH) as described below.

3.3.2.2 Effects of LPS on Fos expression and ADM mRNA expression

The vena cavae were cannulated and exteriorized as described previously (Krukoff et al.,1995) and rats were allowed to recover from surgery. Five days later, the venous lines were flushed with heparinized saline (10 IU/ml) and the rats were allowed to rest for one hour. Rats in the experimental group (n=4) received intravenous (iv) injections of LPS (100 μg/kg; Yang et al., 1999) and the control rats (n=4) received iv injections of saline in an equal volume. Four hours after the injection, rats were deeply anesthetized, and their brains were fixed as described above and stored in 20% sucrose overnight at 4°C. Coronal sections (40 μm) of the brainstem and forebrain were cut in a cryostat and collected in phosphate-buffer saline (PBS) for combined Fos immunohistochemistry and ISH as described below.

3.3.2.3 Effects of restraint stress on ADM mRNA expression

Rats (n=4) were placed in a well-ventilated Plexiglass restraint chamber as described previously (Krukoff et al., 1999a; Krukoff et al., 1999b). Rats were restrained for one hour and allowed to rest for one hour alternately for four hours. Control rats (n=4) were allowed to rest for four hours. Rats were then deeply anesthetized and brains were fixed, cut, thaw-mounted onto Superfrost®/Plus slides, and stored at -70°C for later ISH as described below.

3.3.2.4 Effects of dehydration on ADM mRNA expression

Rats in the experimental group (n=4) were deprived of water for 24 hours, while the control rats were given free access to water. Rats were then deeply anesthetized and brains were fixed, cut, thaw-mounted onto Superfrost®/Plus slides, and stored at -70°C for later ISH as described below.

3.3.3 In situ hybridization (ISH)

A 1.35 kb cDNA encoding the ADM precursor, preproADM (ppADM) was kindly provided by Dr. E.J. Taparowsky (Purdue University). Post translational modification of ppADM results in the formation of ADM, and proadrenomedullin N-terminal 20 peptide (PAMP). ^{35}S-labeled RNA probes were generated from this ppADM cDNA. Antisense probes were transcribed with T7 polymerase (Promega) from plasmids linearized with *Bam*HI; sense probes were transcribed with T3 polymerase (Promega) from plasmids linearized with *Apa*I.

In situ hybridization (ISH) was performed as described previously (Krukoff et al.,1999a,b). In brief, frozen sections were brought to room temperature and allowed to dry, then fixed in 4% paraformaldehyde in PBS for 20 min, and rinsed twice in PBS.

Slides were treated with proteinase K buffer for 7.5 min, followed by a rinse in PBS for 10 min and treatment with 4% paraformaldehyde in PBS for 4 min. Slides were placed in acetic anhydride in 0.1M triethanolamine for 10 min, followed by 70% ethanol with NaAC, 80% ethanol with NaAC, 95% ethanol twice, and allowed to air dry.

Each slide was hybridized overnight at 45°C in humid chambers with approximately 3×10^6 CPM of the labeled probe in 65 µl hybridization buffer (50% formamide, 10% dextran sulfate, 1×Denhardt's solution). The next day, slides were moved to room temperature (RT) for 1 hour and the sections were rinsed twice in 4×SSC with 2-mercaptoethanol (0.1%) for 10 min each, followed by two rinses in 4×SSC. Slides were placed in RNase A in STE buffer at RT for 30 min, followed by STE buffer alone at RT for 30 min, 2×SSC at 42°C for 40 min, and 0.1×SSC at 65°C for 45 min. Sections were allowed to air dry.

Slides were exposed to X-ray film (X-OMAT AR, Kodak) for 48 hours, dipped in NTB-2 Kodak photographic emulsion (diluted 1:1 with water), exposed for 14 days, and developed for autoradiography. Slides were Nissl-stained in 0.5% cresyl violet (C 1791, Sigma) for 2 min and rinsed with water for 1 min, and then dehydrated through graded ethanols. Slides were then immersed in xylene and cover slipped. Sections were examined with light- and dark- field microscopy to determine the anatomical distribution and relative quantity of mRNA expression indicated by the density of the silver grains covering the sections.

3.3.4 Combined Fos immunohistochemistry and ISH

The protocol for combined Fos immunohistochemistry and ISH has been described previously (Krukoff et al., 1999a, b). Sections were incubated overnight at room temperature with rabbit anti-c-*fos* antiserum (#PC38, Oncogene, Uniondale, NY, USA) in PBS. According to the supplier's specifications, this antibody recognizes Fos and Fos-related protein; therefore, the staining we have obtained will be described as Fos-like immunoreactivity (FLI). The following day, tissues were processed using the avidin-biotin immunoperoxidase method (ABC Vecta Stain Kit; Vector laboratories, Burlingame, CA, USA). FLI was visualized as a brown reaction product with the chromagen diaminobenzidine (#D-5637, Sigma). Sections were mounted onto Superfrost®/Plus slides, allowed to air dry, and stored at -70°C for later ISH processing as described above.

3.3.5 Production of photomicrographs

Photomicrographs were captured with a digitizing scanning camera (DC330, DAGE MTI Inc., Michigan IN) mounted onto the microscope and attached to a Pentium 580 computer. The image editing software, Adobe Photoshop was used to process the images, which were printed with a dye sublimation printer (Kodak 8600).

3.3.6 Analysis

ADM mRNA levels in the brain were examined under dark-field microscopy with a digitizing scanning camera (DC330, DAGE MTI Inc., Michigan IN) coupled to a computer with Image software (Image-Pro Plus, Media Cybernetics, Silver Spring MD). For each area studied, two sections from each brain were analyzed and a circular frame

was used to define the area for quantifying the optical density of positive signal. Optical density was defined as percentage of area covered by silver grain per section (Krukoff et al., 1999a, b; Yang and Krukoff, 2000). Background signal was measured on a nearby area where no positive signal was found, and subtracted from the signal in the examined area.

All data are expressed as means ± SEM (n=4 for each group of rats) and a Student's t-test was used to test for statistical significance between two groups ($P<0.05$).

3.4 RESULTS

3.4.1 Distribution of ppADM mRNA in the rat CNS

The distribution of ppADM mRNA in the rat brain is summarized in Table 1.

Telencephalon: A strong ISH signal was seen in the cerebral cortex and olfactory tubercle. Moderate numbers of ppADM mRNA positive neurons were found in the tenia tecta, bed nucleus, stria terminalis, and septum pallidum. In addition, the caudate putamen, accumbens, ventral pallidum, globus pallidus, and substantia innominata also contained a few ppADM-expressing neurons. A high level of ppADM mRNA was detected in neurons of the hippocampal complex, including the CA1, CA2 and CA3 regions of Ammon's horn (Fig. 1A and A'), dentate gyrus, and subiculum. In the amygdala, numerous moderately labeled cells were seen in the basolateral (Fig. 1B and B') and medial nuclei, while the central nucleus showed some weakly labeled neurons.

Diencephalon: Labeled neurons were found throughout the hypothalamus, with the highest levels of mRNA in the hypothalamic supraoptic nucleus (SON), moderate levels in the preoptic areas, paraventricular (PVN, Fig. 1C and C'), arcuate (ARC, Fig.

1D and D'), and ventromedial (VMH) nuclei and weak signal observed in lateral, suprachiasmatic, periventricular, and mammillary nuclei. The zona incerta also showed a few lightly labeled cells. However, no positive signal was detected in the anterior, lateroanterior or dorsomedial nucleus. In the thalamus, moderately labeled neurons were found in the anteroventral, paraventricular and habenular nuclei. Except for the weakly labeled cells in the anterodorsal, mediodorsal, posteroventral, the rest of the thalamus was unlabeled.

Mes- and metencephalon: The most intense signal was seen in the locus coeruleus (LC, Fig. 2A and A'). Interfascicular and motor trigeminal nuclei contained moderate levels of signal. The cerebellum also revealed a moderate number of cell bodies labeled for ppADM mRNA. A few weakly labeled cells were seen in the ventral tegmental areas, substantia nigra, central periaqueductal gray, superior colliculus, red nucleus, parabrachial nucleus, and abducens nucleus.

Myelencephalon: Highly labeled neurons were found in the dorsal motor nucleus of the vagus (DMX, Fig. 2B and B') and rostroventrolateral reticular nucleus. Moderately labeled neurons were seen in the facial, hypoglossal, ambiguus (Fig. 2C and C'), lateral reticular, paragigantocellular reticular, and inferior olivary nuclei. Weak signal was present over neurons in the raphe obscurus, gracile, and vestibular nuclei, and the nucleus of the solitary tract (NTS, Fig. 2B and B').

Other areas: The circumventricular organs (CVOs) including area postrema (AP, Fig. 2B and B') and subfornical organ (SFO) contained moderate numbers of ppADM- expressing cells. In the spinal cord (Fig. 3A and A'), moderate levels of signal were detected in the dorsal and ventral horns, with the highest levels over neurons

in the intermediolateral cell column (Fig. 3B and B'). In the pituitary gland, a moderate signal was present in the posterior lobe (PP), but not in the anterior (AP) or intermediate (IP) lobe (Fig. 3C and C').

In control experiments with the sense probe, no positive signal was detected (Fig. 1E and E').

3.4.2 Effects of LPS on FLI and ppADM gene expression (Fig. 4)

In confirmation of earlier studies (Elmquist et al., 1996; Yang et al., 1999), iv injections of LPS markedly stimulated FLI throughout the rat brain, especially in key autonomic regulatory nuclear groups, including both parvocellular (pPVN) and magnocellular PVN (mPVN), LC, NTS, and VLM. Among the regions examined, pPVN, mPVN, SON, ARC, NTS, AP and SFO showed increased numbers of neurons with FLI ranging from 11 to 21 fold, compared with saline controls (data not shown). In contrast, LPS led to significant reduction in the ADM mRNA levels in pPVN (58.9 %), mPVN (40.2 %), SON (37.3 %), AP (50.5 %) and DMX (49.5 %) compared to saline controls, but did not alter ADM mRNA expression in the SFO, ARC, amygdala, VMH and NTS (Fig. 6A and Table 2).

3.4.3 Effects of restraint stress on ppADM gene expression

In restrained rats, significantly lower levels of ppADM mRNA were found in pPVN (50.0 %), mPVN (50.9 %), SFO (34.1 %, Fig. 5), AP (67.0 %), NTS (46.2 %) and DMX (68.8 %) among the examined brain regions, compared to control rats (Fig. 6B and Table 2).

3.4.4 Effects of dehydration on ppADM gene expression

In dehydrated rats, significantly decreased levels of ppADM mRNA were found in only mPVN and SON (46.0 % and 53.1 %, respectively), compared to control rats. (Fig. 6C and Table 2)

3.5 DISCUSSION

3.5.1 Distribution of ppADM mRNA in the rat CNS

The present study describes for the first time the anatomical distribution of neurons throughout the rat CNS which synthesize ppADM mRNA. Generally, the wide distribution of neurons expressing this gene matches the localization of ADM-like immunoreactivity in the rat CNS (Serrano et al., 2000). Our findings are also consistent with results from dissected rat brain regions showing that ppADM mRNA is highly expressed in the pituitary and hypothalamus, and is detectable in the cerebral cortex, cerebellum, brainstem, and hippocampus (Hwang and Tang, 1999).

Although ppADM mRNA and its IR are closely correlated, mismatches are found in some brain regions. For example, nucleus of the diagonal band of Broca; anterior, lateroanterior, and dorsomedial hypothalamus; anteromedial, laterodorsal, and lateroposterior thalamus; inferior colliculus; and interstitial nucleus do not express ppADM mRNA, but reveal ADM IR (Serrano et al., 2000). It is possible that the slow turnover rate of ADM and relatively large amount of stored peptide make ADM peptide, but not mRNA, detectable. On the other hand, ppADM mRNA is expressed in some areas such as parabrachial nucleus and abducens nucleus where ADM IR has not been

described. Because the peptide-specific post translational processing of ppADM, a precursor peptide, results in the formation of two biologically active peptides, ADM and PAMP, the presence of ppADM mRNA may indicate the synthesis of the ADM precursor, but not ADM.

The widespread distribution of ADM and its specific binding sites (Owji et al., 1995) throughout the CNS suggests multiple biological activities of central ADM. In the cerebral cortex, ADM may be involved in the acute response to ischemia as it has been shown that ADM gene expression and neuronal immunoreactivity are increased in the rat cerebral cortex following ischemia. Furthermore, icv administration of a relatively high dose of ADM exacerbates focal ischemic injury (Wang et al., 1995). Interestingly, several other areas in the forebrain with high levels of ppADM mRNA are related to autonomic functions, including the PVN, SON, amygdala and ARC. The PVN is an integrative center for neuroendocrine, autonomic and behavioral regulation of homeostasis, and consists of two distinct populations of neurons, parvocellular (pPVN) and magnocellular (mPVN) neurons. The pPVN is involved in the regulation of the hypothalamo-pituitary-adrenal (HPA) axis and of autonomic responses; the mPVN participates in the control of the hypothalamo-neurohypophysial system through synthesis of vasopressin (AVP) and oxytocin (OXY) and projection to the posterior pituitary (Swanson and Sawchenko, 1983). The SON is composed of magnocellular neurons with similar efferent projections as the mPVN. The amygdala is a limbic structure with extensive connections to the hippocampus, hypothalamus, and cortex, and is proposed to play a role in the cardiovascular and neuroendocrine stress response (Roozendaal et al., 1992; Sanders et al., 1994). The ventromedial nucleus of the hypothalamus (VMH) is considered as one of

the major brain satiety centers (Swanson, 1987), and is also thought to participate in feedback circuits regulating the HPA axis (Suemaru et al., 1995). Likewise, the ARC has extensive projections to the PVN, and is also implicated in regulation of stress responses (Baker and Herkenham, 1995).

In the brainstem, ADM-producing neurons are found in the NTS, LC, and ventrolateral medulla (VLM), regions whose roles in regulation of autonomic functions are well established. Catecholaminergic neurons are prominent in these brainstem areas and participate in the integration of hypothalamic responses to stress (Pacak et al., 1995). Therefore, ADM may be colocalized and interact with catecholamines in these centers. ADM is also present in the AP and SFO, circumventricular structures which are important for communication between the circulation and the CNS because of their lack of a blood-brain barrier. Whereas AP has been suggested as a site at which ADM acts to influence neuroendocrine and autonomic functions (Samson, 1998; Shan and Krukoff, 2000), the importance of the SFO in ADM signaling requires further investigation.

Finally, the detection of ppADM mRNA in the IML and DMX, sympathetic and parasympathetic preganglionic neurons, respectively, suggests that ADM may influence the activities of these two systems. The evidence for ADM in regulation of the sympathetic activity has been demonstrated by the findings that central administration of ADM increases blood pressure and sympathetic output in anesthetized rats (Takahashi et al., 1994), and that increases in blood pressure and heart rate stimulated by central ADM are abrogated by α-adrenergic blockade in conscious rats (Samson et al., 1998).

In agreement with our findings for ppADM mRNA distribution in autonomic centers, ADM's functional relevance has been suggested by the observations that central

administration of ADM in rats stimulates activity of the the sympathetic nervous system as described above (Takahashi et al., 1994), and inhibits water drinking (Murphy and Samson, 1995), salt intake (Samson and Murphy, 1997), feeding (Taylor et al., 1996), and gastric emptying (Martinez et al., 1997).

In addition to its widespread presence in autonomic related brain regions, ppADM mRNA is also found in sensory and motor neurons of the CNS. ADM belongs to the calcitonin gene-related peptide (CGRP) superfamily which includes CGRP, calcitonin and amylin, and shares functional similarities with CGRP, including potent vasodilatory actions and decreased food and water intake when administered centrally (van Rossum et al., 1997). CGRP is known to play a role in the formation, maintenance and normal function of the neuromuscular junction and is also involved in the spinal transmission of nociceptive stimuli (van Rossum et al., 1997). Whether ADM plays similar roles in sensory and motor neurons remains to be determined.

3.5.2 Modulation of ppADM mRNA gene expression in response to different stressors

Our results show that ppADM gene expression is differentially regulated in autonomic centers of the brain and that each stressor produces its own unique profile of expression. LPS inhibits ppADM gene expression in the pPVN, mPVN, SON, DMX, and AP; restraint stress reduces ppADM mRNA levels in the pPVN, mPVN, SON, NTS, DMX, AP, and SFO; twenty-four hours of water deprivation decreases ppADM gene expression only in the mPVN and SON.

LPS, an immune stressor, is well known to induce an array of peripheral and central responses associated with increased activity of the HPA axis as shown by increased CRF gene expression in the PVN (Kakucska et al., 1993) and circulating corticosterone levels (Besedovsky et al., 1986). Restraint stress, a psychological stressor, elicits characteristic behavioral and physiological responses associated with an increase in the activity of the HPA axis and sympathoadrenal system as shown by increased plasma levels of adrenocorticotrophic hormone (ACTH), corticosterone, adrenaline and noradrenaline (Gomez et al., 1996). Dehydration by water deprivation leads to increased plasma osmolality which activates magnocellular neurons in the PVN and SON to release AVP and OXY into the circulation (Wakerley et al., 1978; Sladek et al., 1985; Windle et al., 1993). In addition, these three stressors induce significant and distinct patterns of neuronal activation of autonomic centers as shown by expression of c-*fos* (Pezzone et al., 1992; Cullinan et al., 1995; Elmquist et al., 1996; Krukoff and Khalili, 1997; Patronas et al, 1998; Krukoff et al., 1999a; Morien et al., 1999; Yang et al., 1999).

Gene expression of ppADM is decreased in the pPVN in LPS-treated and restrained rats compared to their controls. These results are in striking contrast to results for regulation of most other neuropeptides produced in the pPVN (e.g. CRF, AVP, neurotensin, and preproenkephalin) whose gene expression is increased in response to LPS and/or restraint (Lightman and Young, 1987; Bartanusz et al., 1993; Kalin et al., 1994; Juaneda et al., 1999). The central administration of ADM has been reported to activate pPVN neurons as assessed by Fos expression (Serino et al., 1999), although it is difficult to separate the blood pressure-induced neuronal activation from direct activation of pPVN neurons by ADM. The inhibition of basal and CRF-induced ACTH secretion in

cultured pituitary cells (Samson et al., 1995) and of ACTH release in vivo after systemic injections of ADM in sheep (Parke and May, 1995) suggest that ADM has direct inhibitory effects on the pituitary gland. On the other hand, intracerebroventricular injections of ADM have been shown to stimulate (Charles et al., 1998) or to have no effect on (Parkes and May, 1995) ACTH release into the circulation of sheep. Therefore, the central effects of ADM on the HPA axis are not yet clear. However, if ADM's central effects on the HPA axis are indeed opposite to its direct effects on pituitary cells, our results showing that LPS and restraint down-regulate ppADM gene expression in the pPVN after four hours suggest that reduction in ADM production may lead to decreased ACTH secretion and thus restore homeostatic balance in the HPA axis after the early surge in stress-induced ACTH release.

Decreased ppADM gene expression in the mPVN and SON in response to LPS, restraint, and dehydration suggests a role for ADM in regulating neurohypophysial hormones in response to physiological stressors. In particular, recent evidence supports the hypothesis that ADM is involved in the central control of body fluid balance. ADM antisense oligonucleotide treatment in rats was shown to markedly decrease ADM peptide content in the PVN and to lead to exaggerated sodium intake (Samson et al., 1999). Furthermore, central ADM administration inhibits water drinking (Murphy and Samson, 1995). Central ADM administration stimulates Fos expression in mPVN and SON neurons (Serino et al., 1999), but suppresses hypovolemia- or hypernatremia-induced AVP release (Yokoi et al., 1996). AVP is a significant mediator of body fluid balance, and dehydration activates magnocellular neurons to increase AVP synthesis and release into the circulation (Dunn et al., 1973; Zerbe and Palkovits, 1984). Our results

show that stressors suppress ppADM gene expression and suggest, therefore, that decreased ADM production may facilitate increased levels of circulating AVP.

Decreased ppADM gene expression in mPVN and SON also indicates that ADM may affect OXY production in, or release from, magnocellular neurons. Indeed, it has been shown that centrally administered ADM activates predominantly OXY (and fewer AVP) neurons in the mPVN and SON, and increases plasma oxytocin levels in the rat (Serino et al., 1999). Apart from its classical functions in reproductive physiology, OXY has been shown to be released into the plasma in response to psychological stressors (Lang et al., 1983; Jezova et al., 1995; Hashiguchi et al., 1997; Nishioka et al., 1998). Therefore, the reduction in ppADM mRNA in the mPVN and SON which we found in stressed rats may be related to attenuation of levels of circulating OXY.

The area postrema (AP) and subfornical organ (SFO) are CNS sites at which neuropeptides such as angiotensin II, atrial natriuretic peptide, AVP and endothelin act to influence neuroendocrine and autonomic functions (Ferguson and Bains, 1996). *In vitro* electrophysiological observations have shown that AP neurons are highly responsive to ADM (Allen and Ferguson, 1996), and subsequent *in vivo* microinjection studies demonstrated that administration of ADM into the AP leads to increased blood pressure (Allen et al., 1997). More recently, our AP ablation study provided evidence that circulating ADM can access the CNS through the AP to regulate autonomic functions (Shan and Krukoff, 2000). In the present study, ppADM gene expression in AP neurons is decreased following LPS and restraint stress, but is not altered after 24 hours of water deprivation. One possible explanation is that AP neurons with projections to the adjacent NTS produce less ADM to influence autonomic responses to immune and restraint

stressors. Alternatively, because glial cells can also produce ADM (Takahashi et al., 1997), reduced ADM synthesis in glial cells of the AP may influence the activity of AP neurons and thus affect the autonomic responses to these two stressors. The SFO, located in the roof of the third ventricle, has extensive connections to the PVN (McKinley et al., 1990). The SFO has not yet been implicated as a CNS site for ADM interactions nor is it known if ADM neurons in the SFO project to the PVN. Our results showing that ppADM mRNA levels are reduced in the SFO in response to restraint stress do suggest, however, that ADM neurons in the SFO participate in central responses to restraint stress.

It is interesting that, of the three stressors applied in this study, only restraint stress led to a change (decrease) in ppADM mRNA levels in the NTS. Extensive reciprocal connections exist between the NTS, the PVN, and other central autonomic centers (Krukoff et al., 1995; Palkovits, 1999). Our data suggest, therefore, expression of ppADM in the NTS is associated with the autonomic responses that occur within the NTS in response to restraint stress.

We show that ppADM mRNA levels are reduced in the DMX of LPS-treated and restrained rats. While little is known of ADM's role in regulation of parasympathetic activity, ADM has been shown to prevent ethanol-induced gastric injury through vagal-dependent cholinergic pathways (Kaneko et al., 1998). Whether our results are related to gastrointestinal functions or other parasympathetic activities awaits further investigation.

3.5.3 Conclusions

The present study provides a detailed map of neurons which produce ppADM mRNA in the rat CNS and also demonstrates that ppADM gene expression is down-

regulated in autonomic brain regions in response to LPS, restraint, and dehydration. Considering the different central pathways which are activated by these stressors, it is not surprising that the various stressors result in altered ppADM gene expression in different subsets of brain areas examined. Nevertheless, all three stressors cause similar patterns of reduced ppADM gene expression, suggesting that central ADM influences autonomic responses, the HPA axis, and the hypothalamo-neurohypophysial system. Because centrally administered ADM activates neurons in the PVN (Serino et al., 1999), leads to increased sympathetic discharge from the brain (Takahashi et al., 1994), and may stimulate ACTH release from the pituitary (Charles et al., 1998), we hypothesize that physiological stressors lead to attenuation of ADM production in autonomic centers as the animal attempts to re-establish homeostatic balance in these systems.

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TABLE 3-1: Distribution and relative quantity of ppADM mRNA expressing neurons in the rat brain (according to Paxinos and Watson, 1986)

Area	Relative abundance
Telencephalon	
Cerebral cortex	+++
Tenia tecta	++
Corpus callosum	-
Olfactory tubercle	+++
Caudate putamen	+
Accumbens	+
Bed nucleus, stria terminalis	++
Septum nucleus	++
Ventral pallidum	+
Nucleus of the diagonal band (Broca)	-
Globus pallidus	+
Substantia innominata	+
Hippocampus	
CA1, CA2, CA3 regions of Ammon's horn	+++
Dentate gyrus	+++
Subiculum	+++
Amygdala	
Basolateral nucleus	++
Central nucleus	+
Medial neucleus	++
Diencephalon	
Hypothalumus	
Anterior	-
Preoptic	++
Lateral	+
Lateroanterior	-
Suprachiasmatic	+
Paraventricular	++
Periventricular	+
Supraoptic	+++
Arcuate	++
Dorsomedial	-
Ventromedial	++
Zona incerta	+
Thalamus	
Anterodorsal	+
Anteroventral	++
Anteromedial	-
Mediodorsal	+
Laterodorsal	-
Lateroposterior	-

Posteroventral	+
Mammillary nucleus	+
Paraventricular	++
Habenular nucleus	++
Mesencephalon	
Ventral tegmental areas	+
Substantia nigra	+
Central periaqueductal grey	+
Interfascicular nucleus	++
Superior colliculus	+
Inferior colliculus	-
Red nucleus	+
Metencephalon	
Cerebellum	++
Locus coeruleus	+++
Parabrachial nucleus	+
Abducens nucleus	+
Motor trigeminal nucleus	++
Myelencephalon	
Raphe obscurus nucleus	+
Facial nucleus	++
Paragigantocellular reticular nucleus	++
Rostroventrolateral reticular nucleus	+++
Ambiguus nucleus	++
Hypoglossal nucleus	++
Nucleus of the solitary tract	+
Dorsal motor nucleus of the vagus	+++
Lateral reticular nucleus	++
Interstitial nucleus	-
Inferior olivary nucleus	++
Nucleus gracilis	+
Vestibular nucleus	+
Circumventricular organs	
Area postrema	++
Subfornical organ	++
Spinal cord	
Dorsal horn	++
Ventral horn	++
Intermediolateral cell column	+++
Pituitary	
Posterior lobe	++
Anterior lobe	-
Intermediate lobe	-

+++ (strong signal), ++ (moderate signal), + (weak signal), - (no detectable signal)

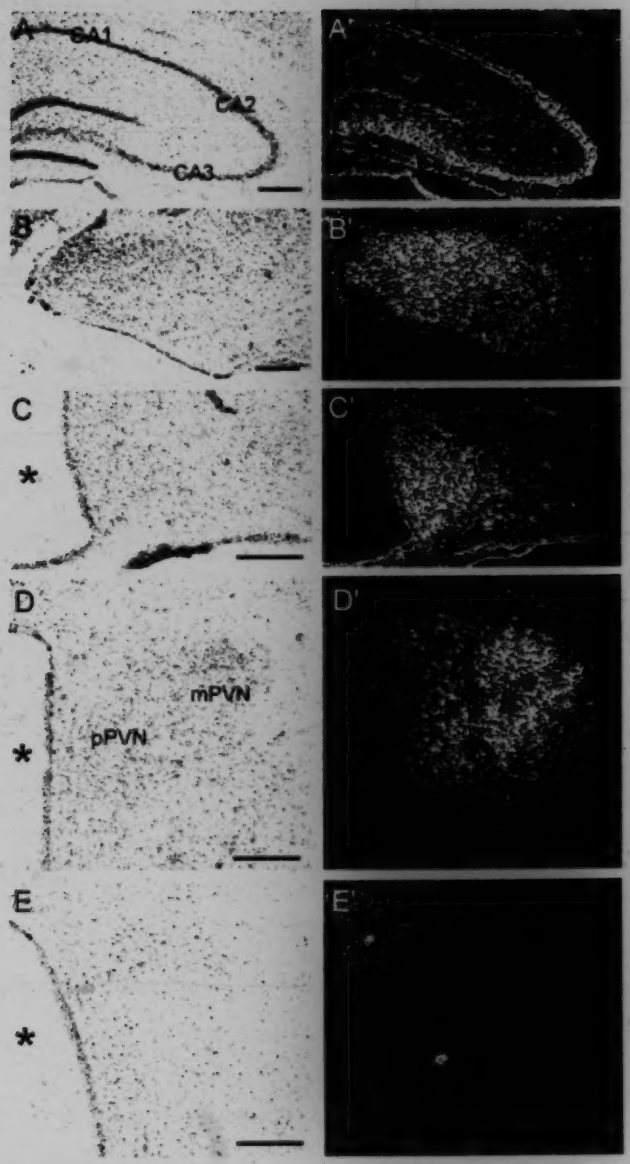

Figure 3-1: Light field (Nissl stain; A, B, C, D, E) and corresponding dark field (A', B', C', D', E') photomicrographs showing the distribution of ppADM mRNA in coronal sections of the rat forebrain. A and A': hippocampus including CA1, CA2, and CA3 regions of Ammon's horn; B and B': basolateral nucleus of amygdala; C and C': arcuate nucleus; D, D', E and E': hypothalamic paraventricular nucleus (PVN) including parvocellular PVN (pPVN) and magnocellular PVN (mPVN). E and E' show no positive signal with the sense probe. *, third ventricle. Scale bars present 400 μm in A and B, 200 μm in C, D, and E.

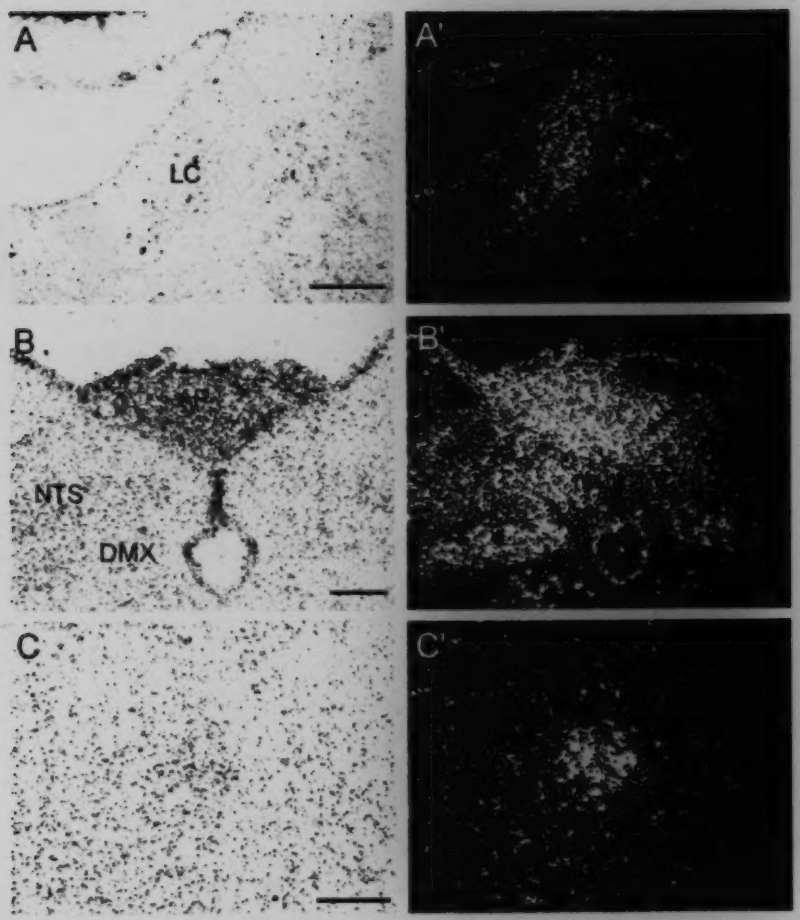

Figure 3-2: Light-field (Nissl stain; A, B, C) and corresponding dark-field (A', B', C') photomicrographs showing the distribution of ppADM mRNA in coronal sections of the rat brainstem. A and A': locus coeruleus (LC). B and B': area postrema (AP), nucleus of the solitary tract (NTS), dorsal motor nucleus of the vagus (DMX); C and C': nucleus ambiguus; Scale bars present 200 μm in A, B and C.

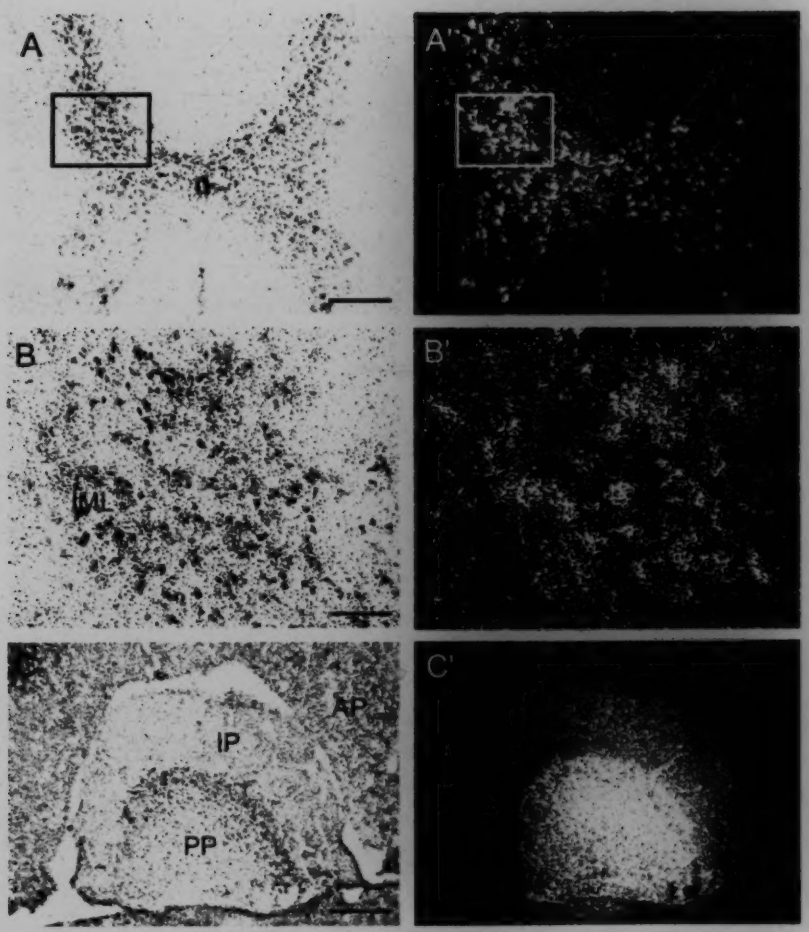

Figure 3-3: Light-field (Nissl stain; A, B, C) and corresponding dark-field (A', B', C') photomicrographs showing the distribution of ppADM mRNA in transverse sections of the rat spinal cord and in the posterior pituitary. A and A': thoracic spinal cord; the rectangles in A and A' include the intermediolateral cell column (IML) and are enlarged in B and B'; C and C' show the pituitary gland including the anterior lobe (AP), intermediate lobe (IP), and posterior lobe (PP). Scale bar presents 200 μm in A, 50 μm in B and 400 μm in C.

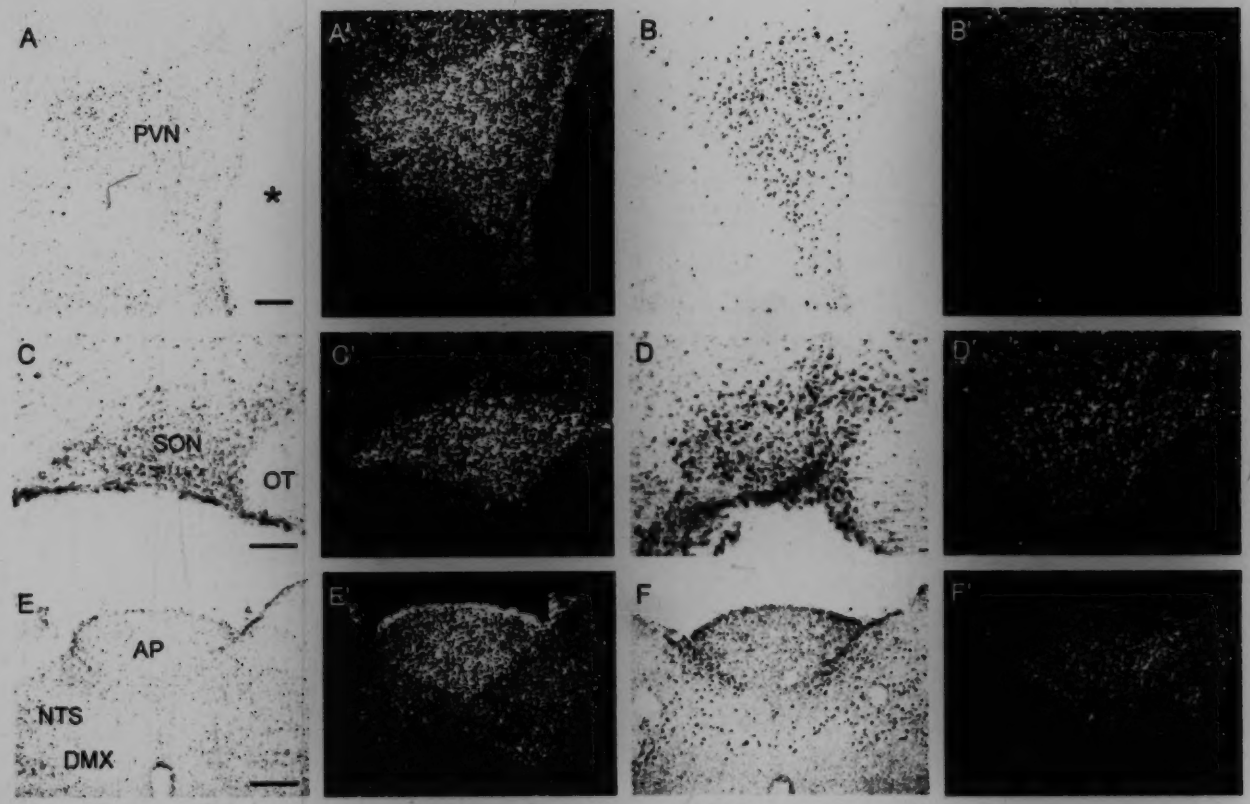

Figure 3-4: Photomicrographs of hypothalamic paraventricular nucleus (PVN, A, A', B, B'), hypothalamic hypothalamic supraoptic nucleus (SON, C, C', D, D'), and dorsal medulla through the area postrema (AP), nucleus of the solitary tract (NTS), and dorsal motor nucleus of the vagus (DMX) (E, E', F, F') from control rats (A, A', C, C', E, E') and LPS treated rats (B, B', D, D', F, F'). Neurons labeled for FLI are shown with light field (A, B, C, D, E, F) and ppADM mRNA is shown with dark field (A', B', C', D', E', F'). *, third ventricle. OT, optic tract. Scale bar in A is for A, A', B, B' and presents 100 μm; bar in C is for C, C', D, D' and presents 100 μm; bar in E is for E, E', F, F' and presents 200 μm.

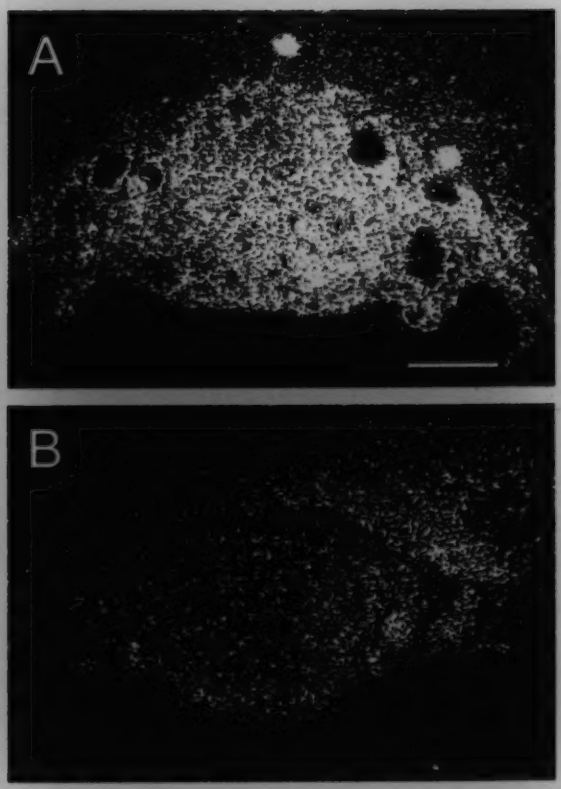

Figure 3-5: Dark field photomicrographs of subfornical organ (SFO) from a control rat (A) and a restrained rat (B) showing hybridization signals for ppADM mRNA. Scale bar in A is for A and B and presents 200 μm.

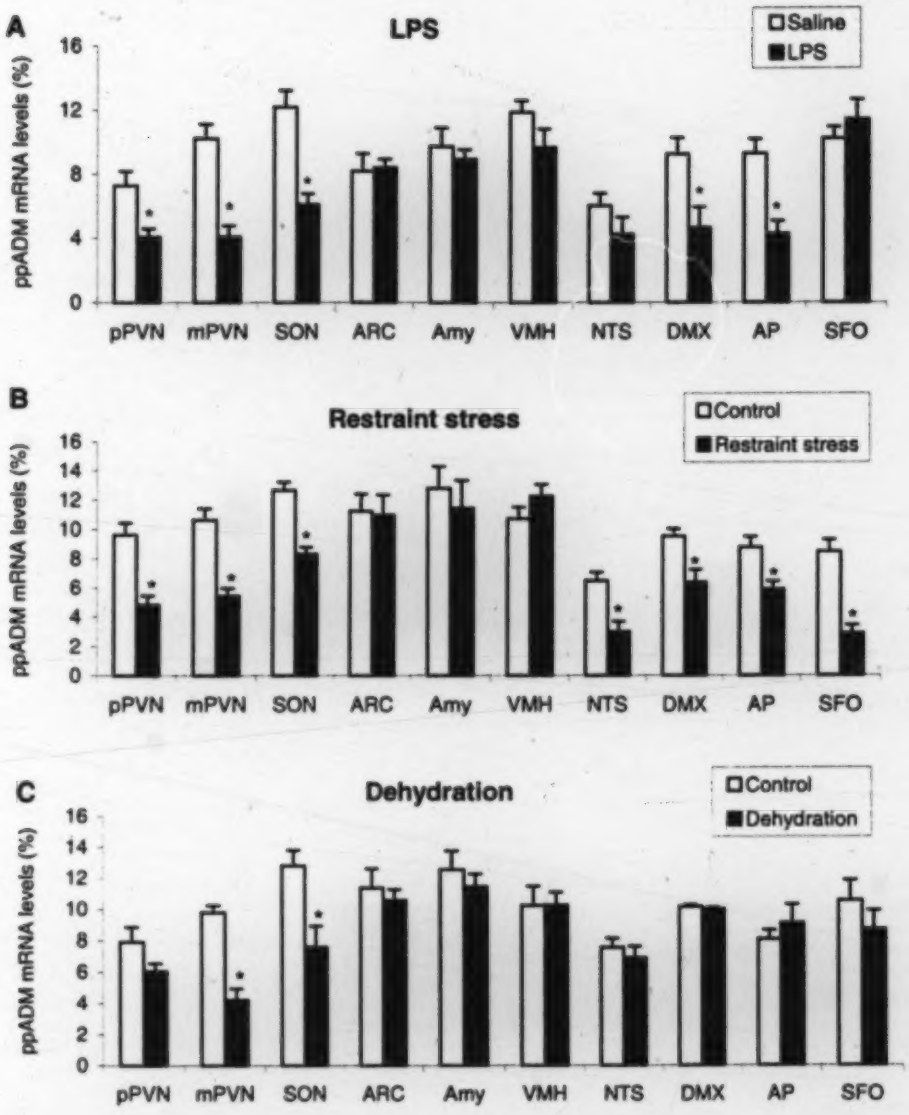

Figure 3-6: Quantitative effects of LPS (A), restraint stress (B) and dehydration (C) on ppADM mRNA levels within autonomic centers. Regions examined include parvocellular hypothalamic paraventricular nucleus (pPVN), magnocellular PVN (mPVN), hypothalamic hypothalamic supraoptic nucleus (SON), arcuate nucleus (ARC), basolateral nucleus of amygdala (Amy), ventromedial nucleus of the hypothalamus (VMH), nucleus of the solitary tract (NTS), dorsal motor nucleus of the vagus (DMX), area postrema (AP), and subfornical organ (SFO). Each column represents the mean ± SEM (n = 4 in each group); * $p < 0.05$, significant difference between groups of rats.

CHAPTER 4

Stimulatory Effects of Intracerebroventricular ADM on the Hypothalamo-pituitary-adrenal Axis and the Sympathetic Nervous System

(The results in this chapter have not yet been published, and are used with permission from my supervisor Dr. T.L. Krukoff.)

4.1 ABSTRACT

It is known that adrenomedullin (ADM) acts centrally to increase blood pressure and inhibit drinking, feeding and salt appetite. This study was designed to investigate the effects of intracerebroventricular (icv) injections of ADM on neuroendocrine and autonomic functions in conscious rats, and to determine whether nitric oxide (NO) may be involved in these responses. Radioimmunoassay (RIA) was used to measure plasma adrenocorticotrophic hormone (ACTH), Fos immunohistochemistry was used to identify activated neurons, nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry was used to localize NO-producing neurons, and in situ hybridization (ISH) was used to quantitate mRNA levels of corticotropin-releasing factor (CRF) in the parvocellular hypothalamic paraventricular nucleus (pPVN), oxytocin (OXY) and vasopressin (AVP) in the magnocellular PVN (mPVN), and tyrosine hydroxylase (TH) in the locus coeruleus (LC) and nucleus of the solitary tract (NTS). Concentrations of nitrate/nitrite were used to measure levels of NO in the hypothalamus and brainstem. Our results showed that icv injections of ADM (2 nmol/kg) in conscious rats increased mean arterial pressure (MAP), plasma ACTH concentrations, and NO production in the hypothalamus, activated neurons in autonomic centers including PVN and LC, and augmented TH mRNA levels in the LC. These results suggest that centrally administered ADM stimulates the activity of the hypothalamo-pituitary-adrenal axis (HPA) axis, stimulates sympathetic functions likely by activating central autonomic pathways, and that these responses may be modulated by NO produced in the hypothalamus.

4.2 INTRODUCTION

ADM, initially isolated from human pheochromocytoma, is a 52 amino-acid peptide and acts as a potent peripheral vasodilator (Kitamura et al., 1993a). ADM is encoded by the preproadrenomedullin (ppADM) gene, which contains the sequence of both ADM and preproadrenomedullin N-terminal 20 peptide (PAMP) (Kitamura et al., 1993b). Since its discovery, ADM immunoreactivity (IR), mRNA and receptors recognizing ADM have been found, not only in peripheral tissues, but also in the brains of human and rat (Ichiki et al., 1994; Owji et al., 1995; Oliver et al., 1998). Apart from hypotensive, diuretic and natriuretic effects in the periphery (Kitamura et al., 1993a; Samson et al., 1995; Vari et al., 1996), ADM also acts centrally to increase blood pressure (Takahashi et al., 1994), and to inhibit water (Murphy and Samson, 1995), salt (Samson and Murphy, 1997), and food (Taylor et al., 1996) intake.

Recent anatomical studies have shown that both ADM IR (Serrano et al, 2000) and mRNA (Shan and Krukoff, in revision) are widely distributed throughout the rat central nervous system (CNS), with high levels in autonomic centers such as hypothalamic paraventricular nucleus (PVN), hypothalamic supraoptic nucleus (SON), locus coeruleus (LC), intermediolateral cell column of the spinal cord (IML). We have also observed that ADM gene expression is altered in autonomic brain regions including PVN in response to lipopolysaccharide (LPS), restraint, and dehydration (Shan and Krukoff, in revision). These findings suggest that endogenous brain-derived ADM participates in the regulation of autonomic and neuroendocrine functions. However, how central ADM affects these functions is unknown.

The PVN is an important integrative center for autonomic nervous system and neuroendocrine functions, comprising parvocellular and magnocellular neurons. Parvocellular neurons either produce corticotropin-releasing factor (CRF) to regulate the HPA axis or affect sympathetic outflow with projections to the brainstem and spinal cord including nucleus of the solitary tract (NTS) and LC. Magnocellular neurons synthesize vasopressin (AVP) or oxytocin (OXY) to regulate the fluid and electrolyte balance (Swanson and Sawchenko, 1983). The LC and NTS, located in the brainstem, contain abundant catecholaminergic neurons, supply extensive projections to other autonomic centers including the PVN (Swanson and Hartman, 1975; Swanson et al., 1981), and are considered to be important central sites which regulate autonomic functions. TH, the rate-limiting enzyme in catecholamine (CA) biosynthesis, is abundantly present in the LC and NTS (Kalia et al., 1985), and thus is routinely used as a marker of synthesis of CA.

Nitric oxide (NO), a gaseous neurotransmitter in the CNS, has been implicated in critical processes of the brain which maintain body homeostasis (Krukoff, 1999). Our earlier studies demonstrating that NO neurons are found in many autonomic centers including PVN and SON (Krukoff and Khalili, 1997), and that NO not only participates in different types of stress responses, including immune (Yang et al., 1999) and restraint stress (Krukoff and Khalili, 1997), but also the regulation of BP (Petrov et al., 1995), provide evidence for NO in the regulation of neuroendocrine and autonomic functions. In the periphery, ADM-induced vasodilation is believed to be mediated by NO (Feng et al., 1994; Hirata et al., 1995; Hayakawa et al., 1999), but whether NO also contributes to regulating the HPA axis and autonomic responses induced by central ADM remains unknown.

The present study was designed to assess physiological effects, neuronal activation, and gene expression of CRF, OXY, AVP, and TH in response to icv injections of ADM, and to determine whether NO may be involved in these responses. Blood pressure was monitored during all experiments, RIA was used to measure plasma ACTH, Fos immunohistochemistry was used to identify activated neurons, NADPH-d histochemistry was used to localize NO-producing neurons, in situ hybridization (ISH) was used to examine mRNA levels of CRF in the pPVN, OXY and AVP in the mPVN, and TH in the LC and NTS, and concentrations of nitrate/nitrite were used to determine the production of NO in the hypothalamus and brainstem.

4.3 MATERIALS AND METHODS

4.3.1 Animals

Male Sprague-Dawley rats (250-300 g) were purchased from the Biological Animal Center, University of Alberta. They were housed in a 12:12 hour light/dark cycle at 22°C and given free access to food and water. All experimental protocols were approved by the local Animal Welfare Committee.

4.3.2 Surgical procedures

Rats were anesthetized with sodium pentobarbital (50 mg/kg; i.p.; Somnotol, M.T.C. Pharmaceuticals, Hamilton, Canada) and icv cannulation was performed as described previously (Yang and Krukoff, 2000). A stainless steel guide cannula (22 gauge, 6mm) was fixed in a stereotaxic frame, and an internal cannula connected to a microsyringe was inserted into the guide cannula to protrude 1mm beyond the tip of the

guide cannula. Rats were placed in the stereotaxic frame, and the guide cannula was implanted into the right lateral cerebral ventricle according to the following coordinates: 1mm posterior, 2mm lateral to the bregma, 4.1 mm below the cerebral surface. The proper placement was verified by observing the outflow of CSF when withdrawing the microsyringe. The guide canuula was fixed with three screws on the skull and dental cement, and the internal cannula was replaced with a dummy cannula to close the guide cannula. In the same surgical session, the descending aorta was cannulated and exteriorized as described previously (Krukoff et al., 1995). Wounds were sutured closed and rats were allowed to recover from anesthesia. Rats were handled individually on a daily basis so that they became accustomed to experimental procedures.

4.3.3 Experimental design

Four to five days after the initial surgery, the arterial line was flushed with heparinized saline (10 IU/ml). The arterial line was connected to a pressure transducer to record arterial pressure and the rats were left undisturbed for 1 h before experimentation. Experimental rats received icv injections of rat adrenomedullin (ADM, 2 nmol/kg; #9508, Peninsula Laboratories, Inc; Belmont, CA, USA) dissolved in 10 µl saline; control rats received icv injections of 10 µl saline. Blood samples (300 µl) were withdrawn at 0, 15, 30, 60 min after the icv injection of ADM or saline, collected into tubes containing EDTA, and centrifuged at 6000 rpm for 10 min. Blood volume was replaced with saline immediately after each blood sample was obtained. Plasma samples were stored at –70 °C for later ACTH RIA as described below. At 60 min after icv injections, rats were deeply anesthetized with Somnotol and decapitated. In early studies, we found that

anethesia with Somnotol in short duration (<5 min) has no effect on NO production in the brain. Hypothalamus and brainstem were dissected from each brain, homogenized in 750μl PBS, and centrifuged at 10 kg for 20 min. The supernatant was ultracentrifuged at 100 kg for 15min, and the final supernatant was stored at –20 °C for a nitrate/nitrite assay as described below.

Another set of rats were divided into three groups (n=4 for each group):

Group 1 (ADM, 2 inj): ADM given at 0 min, ADM given at 120 min (to enhance probability of detecting changes in mRNA levels);

Group 2 (ADM, 1 inj): ADM given at 0 min, saline given at 120 min;

Group 3 (Saline): saline given at 0 min, saline given at 120 min.

Blood sampling was performed at 0, 60, 120, 180, 360 min after the first icv injections of ADM or saline, as described above. At the end of four hours, rats were deeply anesthetized and perfused transcardially with 200 ml saline followed by 500 ml ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). The brains were removed, postfixed in half-strength fixative and 10% sucrose for 1 h, and stored in 20% sucrose overnight at 4°C. Coronal sections (40 μm) of the brain were cut in a cryostat. Three sets of sections from each brain were thaw-mounted onto Superfrost/Plus slides, air dried and stored at -70°C for later in situ hybridization (ISH), and one set were collected in PBS for Fos immunohistochemistry as described below.

4.3.4 Plasma ACTH assay

Plasma ACTH concentrations were measured using a commercially available radioimmunoassay (RIA) kit (ICN Pharmaceuticals, Inc., Costa Mesa, CA). Briefly, 100

μl of plasma were incubated overnight at 4 °C with 100 μl of ACTH antiserum and hACTH-^{125}I as a tracer. The ACTH antibody recognizes ACTH $^{1-134}$, ACTH $^{1-124}$, but does not crossreact with h β-endorphin, h β- and α-lipotropin, or h α- and β- melanocyte-stimulating hormone (<1 %). After incubation, 500 μl of precipitant solution was added, and the pellet was obtained by centrifuging at 1000 g for 15 min. Radioactivity in the pellet was counted in a gamma counter. The limit of detection for ACTH is 10 pg/ml. The intra-assay variation is 5.0 %, and inter-assay variation is 7.3 %.

4.3.5. Nitrate/Nitrite colorimetric assay

The total concentrations of nitrate/nitrite, the final products of NO, were determined by using colorimetric assay (Cayman Chemical, Ann Arbor, MI). This assay was performed as described previously (Yang and Krukoff, 2000). In brief, the frozen supernatant samples were defrosted and filtered through a 30 kDa molecular weight cut-off filter (Millipore, Bedford, MA). Samples (80 μl) were incubated with 10 μl Enzyme co-factor and 10 μl Nitrate reductase for one hour at room temperature. After incubation, 50 μl of Griess Reagent R1 and R2 were added, and the amount of colored product was examined by the absorbance at 540nm using the plate reader (MTX Lab System Inc., Mclean, VA).

4.3.6 Fos immunohistochemistry

Sections of brainstem and forebrain were incubated overnight at room temperature with rabbit anti-c-*fos* antiserum (#PC38, Oncogene, Uniondale, NY, USA) diluted 1:4000 in 0.3% Triton X-100/phosphate-buffered saline (PBS, pH 7.2). According

to the supplier's specifications, this antibody recognizes Fos and Fos-related protein; therefore, the staining we have obtained will be described as Fos-like immunoreactivity (FLI). The following day, tissues were processed using the avidin-biotin immunoperoxidase method (ABC Vecta Stain Kit; Vector laboratories, Burlingame, CA, USA). FLI was visualized as a brown reaction product with the chromagen diaminobenzidine (#D-5637, Sigma). After a rinse in PBS, sections were either stained for NADPH-d as described below, or mounted onto Superfrost/Plus slides and stored at -70°C for later in situ hybridization (ISH) as described below.,

4.3.7 NADPH-d histochemistry

Sections with FLI staining were incubated for 20 minutes at 45°C in a solution containing 1 mg/ml β-NADPH (#N-1630, Sigma), 0.3 mg/ml nitroblue tetrazolium (#N-6876, Sigma) dissolved in dimethyl sulfoxide (#D-8779, Sigma), and 0.6% Triton X-100/PBS. Sections were mounted onto glass microscope slides, coverslipped with Elvanol (Moviol, Calbiochem Corporation, La Jolla, CA, USA; dissolved in 40 ml PBS and 20 ml glycerol), and examined with a Zeiss light microscope.

4.3.8 In situ hybridization for CRF, AVP, OXY and TH mRNA

^{35}S-labeled RNA probes for CRF were generated from a 770 bp (from +1283 to +2048) CRF cDNA fragment kindly provided by Dr. R. M. Demo, The University of Michigan, Ann Arbor, MI, and antisense probes were transcribed with T7 polymerase (Promega) from plasmids linearized with *Hind*III. ^{35}S-labeled RNA probes for AVP were generated from a 230 bp AVP cDNA fragment kindly provided by Dr. D. Richter,

University of Hamburg, Hamburg, Germany, and antisense probes were transcribed with SP6 polymerase (Promega) from plasmids linearized with *Hind*III. ^{35}S-labeled RNA probes for OXY were generated from a 190 bp rat OXY cDNA fregment kindly provided by Dr. E. Mohr, University of Hamburg, Hamburg, Germany, and antisense probes were transcribed with SP6 polymerase (Promega) from plasmids linearized with *Eco*RI. ^{35}S-labeled RNA probes for TH were generated from a 1.2 kbp *Eco*RI digest of rat TH cDNA encoding the +14 to +1165 region of TH (Grima et al., 1985) that was subcloned into a pGEM-7Zf transcription vector (Promega Corp., Madison, WI), and antisense probes were transcribed with SP6 RNA polymerase (Promega) from plasmids linearized with *Apa*I.

In situ hybridization (ISH) was performed as described previously (Shan and Krukoff, in revision). In brief, frozen sections were brought to room temperature and allowed to dry, then fixed in 4% paraformaldehyde in PBS for 20 min, and rinsed twice in PBS. Slides were treated with proteinase K buffer for 7.5 min, followed by a rinse in PBS for 10 min and treatment with 4% paraformaldehyde in PBS for 4 min. Slides were placed in acetic anhydride in 0.1M triethanolamine for 10 min, followed by 70% ethanol with NaAC, 80% ethanol with NaAC, 95% ethanol twice, and allowed to air dry. Each slide was hybridized overnight at 45°C in humid chambers with approximately 3×10^6 CPM of the labeled probe in 65 µl hybridization buffer (50% formamide, 10% dextran sulfate, 1×Denhardt's solution). The next day, slides were moved to room temperature (RT) for 1 hour and the sections were rinsed twice in 4×SSC with 2-mercaptoethanol (0.1%) for 10 min each, followed by two rinses in 4×SSC. Slides were placed in RNase A in STE buffer at RT for 30 min, followed by STE buffer alone at RT for 30 min,

2×SSC at 42°C for 40 min, and 0.1×SSC at 65°C for 45 min. Sections were allowed to air dry.

Slides were exposed to X-ray film (X-OMAT AR, Kodak) for 72 hours, dipped in NTB-2 Kodak photographic emulsion (diluted 1:1 with water), exposed for 7 days, and developed for autoradiography. Slides were Nissl-stained in 0.5% cresyl violet (C1791, Sigma) for 2 min, and rinsed with water for 1 min, and then dehydrated through graded ethanols. Slides were then immersed in xylene and cover slipped. Sections were examined with light- and dark- field microscopy to determine the anatomical distribution and relative quantity of mRNA expression indicated by the density of the silver grains covering the sections.

4.3.9 Production of photomicrographs

Photomicrographs were captured with a digitizing scanning camera (DC330, DAGE MTI Inc., Michigan IN) mounted onto the a microscope and attached to a Pentium 580 computer. The image editing software, Adobe PhotoShop, was used to process the images, which were printed with a dye sublimation printer (Kodak 8600).

4.3.10 Subdivision of nuclei

To aid in analysis, sections from the middle PVN were divided into the following functional divisions: posterior magnocellular (pm), dorsal parvocellular (dp), ventral medial parvocelluar (mp_v), and dorsolateral medial parvocellular (mp_d) divisions, as described previously (Shan and Krukoff, 2000).

To assist in quantification of TH mRNA, sections from the middle NTS was divided into two functionally distinct nuclei: the medial subnuclei of the NTS (mNTS) and the dorsal subnuclei of the NTS (dNTS) (Kalia et al., 1985).

4.3.11 Analysis

For the quantification of FLI labeled neurons, only neurons with visible nuclei were counted. Neurons with FLI were counted in the subdivisions of the middle PVN, SON, ARC, NTS, AP and LC. For each area, 3 to 5 sections were analyzed per rat. Counts were expressed as given values *per section* to eliminate factors related to double counting of neurons.

The quantification of mRNA levels for CRF, OXY, AVP, and TH was performed as described previously (Shan and Krukoff, in revision). Briefly, sections with mRNA signal were examined with Image software under dark-field microscopy. Optical density of the signal, defined as percentage of area covered by silver grain per section, was used to quantify mRNA levels, and the signal in the examined area was corrected for the background signal in the nearby area. For each area studied, two sections from each brain were analyzed. CRF mRNA levels were measured in the dp, mp_v, and mp_d; OXY and AVP mRNA levels were measured in the pm; and TH mRNA levels were measured in the LC, mNTS and dNTS.

All data are expressed as means ± SE. A Student's t-test was employed to determine significance between the two groups. Comparison among three groups was performed by a one-way ANOVA followed by a Student-Neuman-Keuls test. In all cases, $P<0.05$ was considered statistically significant.

4.4 RESULTS

4.4.1 Effects of icv injections of ADM on MAP

Icv injections of ADM (2 nmol/kg) caused rapid and significant increases in MAP of rats (Fig.1). MAP was elevated by10 mmHg at 5 min, and then slowly returned to the baseline. MAP remained significantly elevated compared to controls for 25 min. At 120 min, rats received a second injection of either ADM or saline. The second injection of ADM had similar effects on MAP as the first injection (Fig.1).

4.4.2 Effects of icv injections of ADM on plasma ACTH

Rats receiving icv injections of ADM (2 nmol/kg) showed a marked increase in plasma ACTH by 1.8 fold, 2.7 fold, and 1.9 fold, at 15 min, 30 min and 60 min, respectively, compared to vehicle treated rats (Fig. 2). At 120 min when ACTH levels were back to the baseline, rats received a second injection of either ADM or saline. The second injection of ADM also augmented the ACTH levels at 180 min, but to a less extent than the first injection (187 ±17 vs. 229 ±14 pg/ml) (Fig.2)

4.4.3 Effects of icv injections of ADM on NO production in hypothalamus and brainstem

One hour after ADM injections, a significant elevation in the nitrate/nitrite concentration was observed in the hypothalamus (18 ±0.5 vs. 6.7 ±1.6 μM). However, no difference was found in the brainstem between ADM treated rats and controls (3.7 ±1.3 vs. 5.6 ±1.8 μM) (Fig. 3).

4.4.4 Effects of icv injections of ADM on neurons labeled for FLI

Increased numbers of neurons labeled for FLI were found in the PVN, SON, ARC, NTS, AP and LC, in response to the one injection of ADM compared to controls. In addition, rats receiving two injections of ADM showed a greater increase in numbers of neurons with FLI compared to the one injection (Fig. 4). In the PVN, the majority of neurons with FLI was localized in the parvocellular division, with the highest number in the dorsal medial parvocellular (mpd) subdivision. increased numbers of neurons with FLI were also observed in the posterior magnocellular (pm) division of the PVN as well as SON of rats treated with ADM compared to controls, although the numbers were not as great as in parvocellular neurons of PVN.

4.4.5 Effects of icv injections of ADM on mRNA expressions for CRF, OXY, AVP and TH

Four hours after the first icv injection, no detectable difference in CRF mRNA levels was observed in dp, mpv, or mpd division of the PVN following one or two injections of ADM compared to controls. Likewise, neither OXY nor AVP mRNA levels were altered in response to ADM.

Significantly increased levels of TH mRNA were observed in the LC of rats receiving one or two injections of ADM, compared to control rats, but no differences were found between rats receiving one or two injections of ADM. Neither one nor two injections of ADM altered TH mRNA levels in mNTS or dNTS.

4.5 DISCUSSION

The present study was designed to investigate the effects of intracerebroventricular (icv) injections of ADM on neuroendocrine and autonomic functions in conscious rats, and to determine the possible involvement of NO in these responses. Our results show that icv injections of ADM in conscious rats lead to marked increases in the BP and plasma ACTH concentrations, accompanied by an elevation of NO production from the hypothalamus. We also demonstrate that centrally administered ADM activates neurons in the PVN, SON, ARC, AP, NTS, and LC, and augments TH mRNA levels in the LC. These results indicate that icv ADM stimulates both the HPA axis and sympathetic system by activating autonomic neurons or by stimulating the neuronal pathways in which these neurons participate, and that NO of hypothalamic origin may be involved in these responses.

4.5.1 Stimulatory effects on the HPA axis.

Our study shows for the first time that, in conscious rats, icv injections of ADM exhibit stimulatory effects on plasma ACTH levels, consistent with the icv infusion study performed in conscious sheep (Charles et al., 1998). In contrast, another group reported that icv infusions of ADM into conscious sheep had no effect on the plasma ACTH (Parkes and May, 1995). This discrepancy has been suggested to be due to the differences in the numbers of samples and time points examined (Charles et al., 1998). On the other hand, the observations that ADM inhibits basal and CRF-induced ACTH secretion in cultured pituitary cells in a dose-dependent manner (Samson et al., 1995), and that in vivo systemic injections of ADM decrease ACTH and cortisol release in sheep

(Parke and May, 1995) provide evidence for direct inhibitory effects of ADM on the pituitary. Therefore, whereas central ADM increases plasma ACTH levels, systemic ADM acts on the pituitary to decrease ACTH secretion. These opposite effects indicate that icv ADM acts at the level of hypothalamus to affect activity of the HPA axis.

In the hypothalamus, the PVN is considered as a critical structure to integrate the neuroendocrine and autonomic functions (Swanson and Sawchenko, 1983). Our current data demonstrate that icv administrations of ADM induce FLI expression in the PVN, in agreement with the earlier study (Serino et al., 1999). As expected, we also show that the majority of activated PVN neurons is found in the dorsolateral medial parvocellular division (mp_d), the location of neurons associated with the activity of the HPA axis. (Swanson and Sawchenko, 1983), suggesting that centrally administered ADM acts on the PVN to affect the ACTH release. However, whether central ADM can act on the PVN directly, or effects are mediated through activation of afferent neuronal pathways innervating the PVN neurons remains an open question, as no information regarding the distribution of the ADM specific receptor in the rat CNS is available.

CRF, produced mainly in the parvocellular PVN, regulates the HPA axis by stimulating ACTH release from the pituitary gland and subsequent release of cortisol from the adrenal gland. Interestingly, ADM has been reported to be present in the PVN and the fiber tract of ME where CRF is produced and released (Ueta et al., 1995; Ueta et al., 1999; Serrano et al, 2000). Furthermore, functional studies have shown that ADM shares some similar central actions with CRF, including inhibition of food intake, protection against ethanol induced gastric injury, and increased sympathetic activity and blood pressure (Owens and Nemeroff, 1991; Takahashi et al., 1994; Taylor et al., 1996;

Kaneko et al., 1998). These results, together with our findings that icv ADM elevates plasma ACTH levels and activates the PVN neurons, suggest that centrally administered ADM acts on the PVN to regulate the HPA axis probably by interacting with CRF neurons. However, because our results show that CRF mRNA levels are not altered in any subdivision of the pPVN neurons after four hours of icv injections of ADM, it is reasonable to assume that stimulatory effects of icv ADM on the HPA axis are largely due to release of CRF from PVN neurons.

4.5.2 Stimulatory effects on the sympathetic system

Our present results show that icv injections of ADM induce increases in MAP, in accordance with the data from other groups conducted on rats and rabbits (Takahashi et al., 1994; Saita et al., 1998; Matsumura et al., 1999). In addition, centrally administered ADM has been reported to enhance heart rate in conscious rats and rabbits (Takahashi et al., 1994; Matsumura et al., 1999), and to elevate abdominal sympathetic discharge in anesthetized rats (Takahashi et al., 1994) and the renal sympathetic nerve activity in conscious rats (Saita et al., 1998). Furthermore, peripheral administration of phentolamine, an α-receptor antagonist, blocks the elevation of MAP induced by central ADM in conscious rats (Samson et al., 1998). Taken together, these findings indicate that ADM acts through central mechanisms to stimulate sympathetic functions.

Our present data also show that, icv injections of ADM activate neurons in the dp and mp_v subdivisions of the PVN, AP, NTS, and LC, and all of these areas are associated with central cardiovascular regulation (Swanson and Sawchenko, 1983; Kalia et al., 1985; Aston-Jones et al., 1991; Ferguson and Bains, 1996). AP, a circumventricular

organ, has been considered as a major candidate for the action site of ADM. In favor of this view are the observations that ADM exhibits direct excitatory effects on AP neurons in vitro (Allen and Ferguson, 1996), and that microinjections of ADM into the AP result in increased blood pressure (Allen et al., 1997). Further support is provided from our earlier findings that iv injections of ADM activate AP neurons, and that the ablation of AP attenuates the neural activation in the PVN in response to systemic ADM (Shan and Krukoff, 2000). Considering that AP is immediately adjacent to the NTS, and provides dense projections to the NTS where neurons are reciprocally connected with PVN and LC neurons (Palkovits, 1999), it seems likely that activation of neurons in the NTS, LC and PVN in response to central ADM originates, at least in part, at the AP.

Unlike the mp_d subdivision of the PVN, which is related to the activity of HPA axis as described above, the dp and mp_v subdivisions are considered to regulate autonomic functions by projecting to other autonomic centers in the brainstem including the NTS and LC, and spinal cord including the intermediolateral cell column of the spinal cord (IML) (Swanson and Sawchenko, 1983).

Similarly, the NTS and LC, brainstem CA cell groups, have abundant afferent projections to the PVN (Swanson et al., 1981), and are reciprocally connected with PVN neurons. The LC contains the major population of noradrenergic (NE) neurons in the brain with widespread projections throughout the CNS, and is believed to be important in processes such as attention, arousal, and cardiovascular regulation (Aston-Jones et al., 1991). The NTS has been implicated in integrating a variety of autonomic functions. For example, the medial NTS (mNTS) is involved in the regulation of gastrointestinal and

cardiovascular functions, and the dorsal NTS (dNTS) participates in chemoreflexes and baroreflexes (Kalia et al., 1985).

Finally, the PVN, NTS, and LC project caudally to the IML (Palkovits, 1999). Therefore, based on our observations of activation of these neurons and of increased blood pressure after icv injections of ADM, it is likely that the activation of descending pathways to the IML contributes to the stimulatory effects of central ADM on the sympathetic system. It is also possible that the activation of these regions is the consequence of elevated blood pressure, since changes in blood pressure have been shown to induce Fos expression in these cardiovascular regulatory centers (Li and Dampney, 1994; Chan and Sawchenko, 1994).

Additionally, we show that icv injections of ADM elevate TH mRNA levels in the LC, but not in any division of the NTS. These results, together with our finding that icv ADM activates LC neurons, indicate that icv ADM stimulates LC neurons to induce the synthesis of CA.

4.5.3 Effects on the hypothalamo-neurohypophysial system.

ADM has been proposed to play a physiological role in the regulation of the fluid and electrolyte homeostasis (Samson, 1999). In support of this view, icv ADM has been reported to inhibit water (Samson and Murphy, 1997) and salt (Samson and Murphy, 1997) intake, and to exert diuretic and natriuretic actions (Israel and Diaz, 2000). Further evidence has been provided from the antisense oligonucleotide study which shows that decreased ADM content in the PVN leads to exaggerated sodium appetite (Samson et al., 1999).

It is well established that neurons in the mPVN and SON produce OXY and AVP and project to the posterior pituitary, thus are involved in the regulation of the hypothalamo-neurohypophysial system (Swanson and Sawchenko, 1983). Interestingly, ADM IR has been found in the mPVN and SON neurons where ADM coexists with OXY or AVP (Ueta et al., 1995), and ADM IR has also been detected in the hypothalamo-neurohypophysial tract as well (Ueta et al., 1999). In addition, our recent in situ hybridization study demonstrated that the ADM gene is highly expressed in the mPVN and SON, and its expression is altered by dehydration (Shan and Krukoff, in revision). These results provide evidence for endogenous brain-derived ADM in the regulation of the hypothalamo-neurohypophysial system.

Our finding that in conscious rats icv injections of ADM activate mPVN and SON neurons is consistent with a previous study (Serino et al., 1999). Serino et al. also demonstrated that icv injections of ADM increase plasma OXY levels (Serino et al., 1999). In addition, they found that icv ADM preferentially activates OXY-producing neurons in the PVN and SON, rather than AVP-producing neurons (Serino et al., 1999). Moreover, recent extracellular recordings from mPVN cells revealed that the icv administration of ADM excites OXY-secreting cells, but has no effects on AVP-secreting cells (Ueta et al., 2000). These results suggest that centrally administered ADM stimulates OXY-secreting cells in the mPVN and SON to release OXY. However, our current results and those of others (Serino et al., 1999) do not show differences in OXY mRNA levels in the mPVN in response to icv ADM. This finding is not surprising considering the large amount of OXY stores in the mPVN and SON neurons (Gainer and Wray, 1992).

With regard to the AVP, it has been shown that a small number of AVP-producing neurons in the mPVN and SON are activated after icv injections of ADM (Serino et al., 1999). However, centrally administered ADM neither alters plasma AVP levels in conscious rats (Yokoi et al., 1996) or sheep (Parkes and May, 1995; Charles et al., 1998), nor excites AVP-secreting cells in the PVN (Ueta et al., 2000). These results, together with our findings that central ADM does not change AVP mRNA levels in the mPVN, suggest that central ADM is not involved in modulating mPVN neurons to release and synthesize AVP under basal conditions.

4.5.4 The involvement of NO in these responses

This study is the first to implicate NO in neuroendocrine and autonomic responses to icv ADM as shown by increased NO production from the hypothalamus following icv injections of ADM. In the hypothalamus, the most striking groups of NO-producing neurons are found in the PVN (pPVN and mPVN) and SON (Krukoff, 1999). These anatomical findings suggest that NO regulates endocrine and autonomic functions in response to various stimuli by influencing both the HPA axis and sympathetic system. Regarding the HPA axis, most studies (Brunetti et al., 1993; Rivier and Shen, 1994; Sandi and Guaza, 1995) support the contention that NO plays an inhibitory role in modulating the HPA axis, although evidence also exists for a stimulatory role of NO in the HPA axis (Costa et al., 1993; Lee and Rivier, 1998). Likewise, the modulation of NO on the sympathetic system is also controversial, although the majority of studies agree that NO acts to decrease the sympathetic output to the periphery (Krukoff, 1999). Whether NO exerts inhibitory or stimulatory effects on ADM-induced stimulation of the

HPA axis and sympathetic system will be determined by using NO inhibitors in future studies.

4.5.5 Conclusion

In conclusion, our current study indicates that icv injections of ADM in conscious rats stimulate activity of the HPA axis likely by modulating pPVN neurons to release CRF, and stimulate sympathetic activity by activating central autonomic pathways. Either or both processes may be modulated by NO produced in the hypothalamus. Furthermore, stimulatory effects of central ADM on the HPA axis and sympathetic system are opposite to its peripheral effects on the pituitary and vasculature, prompting us to hypothesize that central ADM might counterbalance its peripheral inhibitory effects to reestablish body homeostasis.

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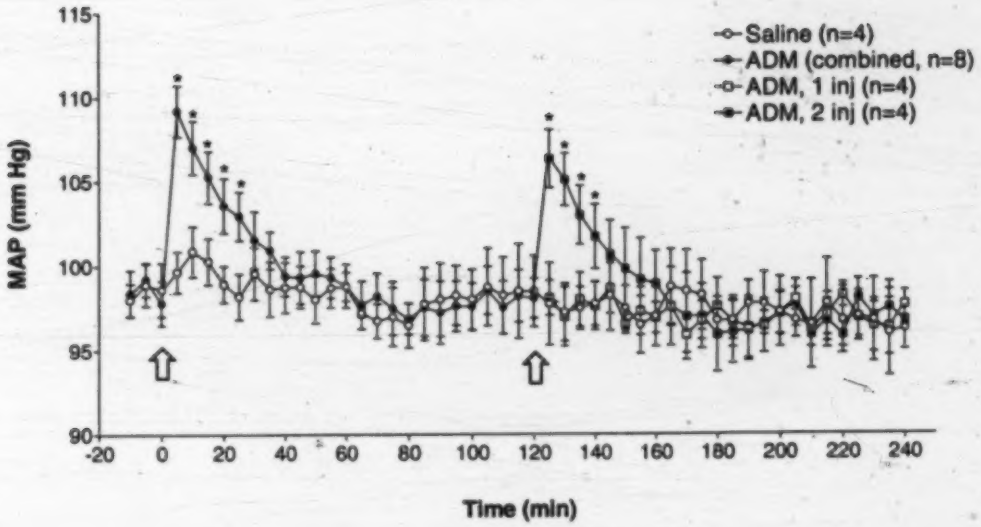

Figure 4-1: Changes in mean arterial pressure in rats receiving one or two intracerebroventricular (icv) injections of adrenomedullin (ADM, 2 nmol/kg), or saline. Icv injections are given at 0 and 120 min. Data from one or two injections of ADM are combined for the first 120 min. Values are expressed as mean ± SE; *, significant difference compared to control ($P<0.05$).

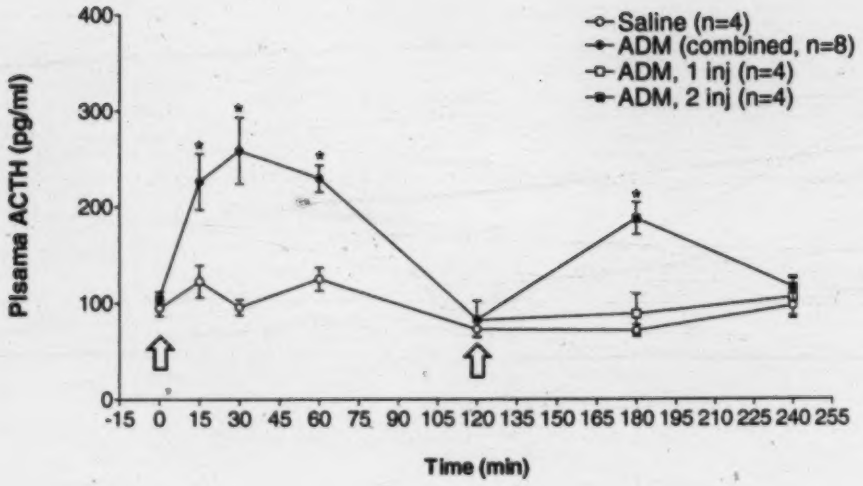

Figure 4-2: Changes in plasma ACTH levels in rats receiving one or two intracerebroventricular (icv) injections of adrenomedullin (ADM, 2 nmol/kg), or saline. Icv injections are given at 0 and 120 min. Data from one or two injections of ADM are combined for the first 120 min. Values are expressed as mean ± SE; *, significant difference compared to control ($P<0.05$).

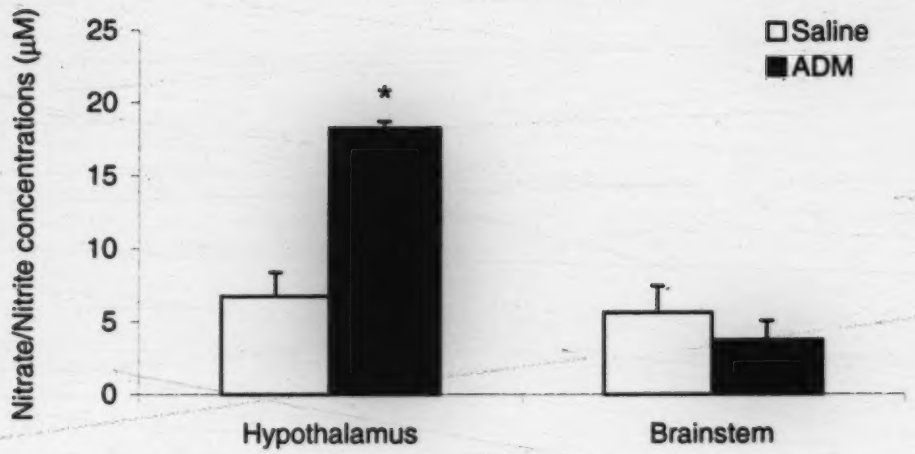

Figure 4-3: Nitrate/nitrite concentrations in hypothalamus and brainstem of rats receiving intracerebroventricular (icv) injections of adrenomedullin (ADM, 2 nmol/kg) or saline. Values are expressed as mean ± SE (n = 4 for each group of rats). *, significant difference compared to control ($P<0.05$).

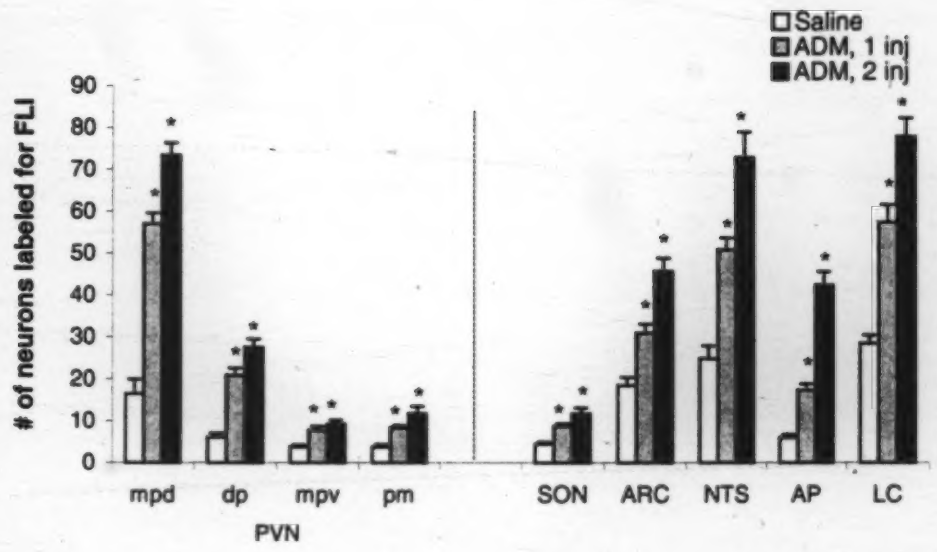

Figure 4-4: Neurons with FLI in the posterior magnocellular (pm), dorsal parvocellular (dp), ventral medial parvocelluar (mp_v), and dorsolateral medial parvocellular (mp_d) divisions of the middle PVN, SON, ARC, NTS, AP, LC from rats receiving one or two intracerebroventricular (icv) injections of adrenomedullin (ADM, 2 nmol/kg), or saline. Values are expressed as mean ± SE (n = 4 for each group of rats); *, significant difference compared to control ($P<0.05$).

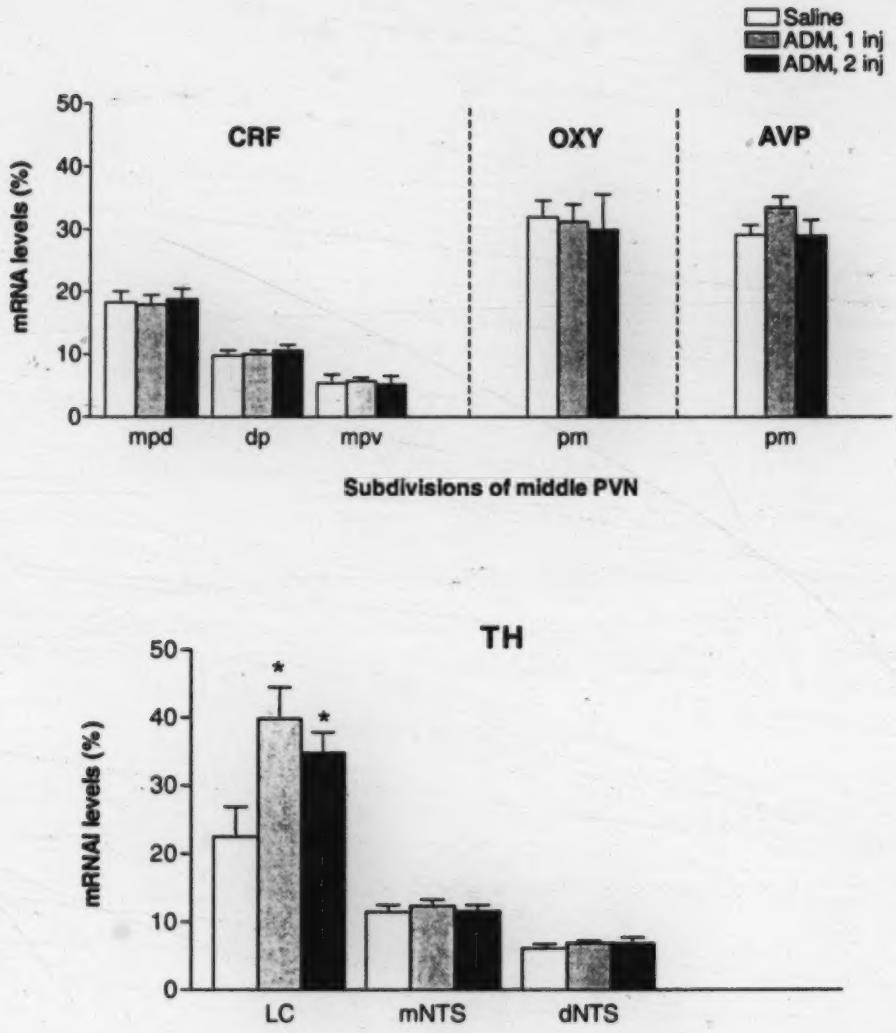

Figure 4-5: Levels of CRF mRNA in dorsal parvocellular (dp), ventral medial parvocelluar (mp_v), and dorsolateral medial parvocellular (mp_d) divisions of the middle PVN; OXY and AVP mRNA in posterior magnocellular (pm) divisions of the middle PVN; and TH mRNA in the LC, mNTS and dNTS in rats receiving one or two intracerebroventricular (icv) injections of adrenomedullin (ADM, 2 nmol/kg), or saline. Values are expressed as mean ± SE (n = 4 for each group of rats); *, significant difference compared to control ($P<0.05$).

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

5.1 DISCUSSION AND CONCLUSIONS

This thesis describes three sets of experiments designed to investigate effects of iv ADM injections on neuronal activation in some autonomic centers, the expression and regulation of ADM mRNA in the rat CNS, and effects of icv ADM injections on neuroendocrine and autonomic functions.

1. We have shown that (1) iv ADM (2 nmol/kg) activates neurons in the PVN, NTS, and AP through a pathway which is not related to the baroreflex. (2) Removal of the AP attenuates the neuronal activation in the PVN. (3) Systemic ADM also increases activation of NO-producing neurons in functionally identified divisions of the PVN. These results provide evidence that the circulating ADM can gain access to the brain through the AP to influence autonomic functions, and that NO might be involved in central autonomic and/or neuroendocrine regulation by systemic ADM
2. Our in situ hybridization experiments have demonstrated that (1) ADM mRNA is widely distributed throughout the rat CNS, with especially high levels in autonomic centers such as hypothalamic paraventricular nucleus (PVN), hypothalamic supraoptic nucleus (SON), locus coeruleus (LC), ventrolateral medulla (VLM), and intermediolateral cell column of the spinal cord (IML). (2) LPS inhibits ADM gene expression in the parvocellular PVN (pPVN), magnocellular PVN (mPVN), SON, dorsal motor nucleus of the vagus (DMX), and area postrema (AP) among examined regions; restraint stress reduces ADM mRNA levels in the pPVN, mPVN, SON, nucleus of the solitary tract (NTS), DMX, AP, and subfornical organ (SFO); 24 hours of water deprivation decreases ADM gene expression only in the mPVN and SON. These findings suggest that endogenous ADM produced within the CNS is involved

in the regulation of the HPA axis, central autonomic functions, and the hypothalamo-neurohypophysial system.

3. We have illustrated that (1) icv injections of ADM (2 nmol/kg) in conscious rats increase MAP, plasma ACTH concentrations, and NO production from the hypothalamus. (2) Neurons are activated in the PVN, SON, ARC, AP, NTS, and LC, and TH mRNA levels are augmented in the LC following icv injections of ADM. These results indicate that icv administered ADM participates in regulating neuroendocrine and autonomic functions by stimulating the HPA axis and the sympathetic system.

The central location of groups of cells expressing ADM specific receptors has not yet been documented, although ADM binding sites have been found in the brain (Sone et al., 1997). However, the AP has been implicated as a potent candidate for the site of action of ADM, as it has been considered as the CNS site for entry of other vasoactive peptides such as angiotensin II, atrial natriuretic peptide, AVP and endothelin (Ferguson and Bains, 1996). In favor of this view, in vitro electrophysiological studies showed that AP neurons are highly responsive to ADM (Allen and Ferguson, 1996), and in vivo observations demonstrated that microinjection of ADM into the AP leads to increased blood pressure (Allen et al., 1997). Further support is provided from our findings that both the iv (Shan and Krukoff, 2000) and icv injections of ADM stimulate neurons in the AP, and that the AP ablation attenuates the activation of the PVN neurons in response to systemic ADM (Shan and Krukoff, 2000).

The PVN is another area of interest in our investigation of how ADM affects autonomic and neuroendocrine functions. Firstly, we have shown that many PVN

neurons are activated following either iv or icv injections of ADM. In the case of systemic ADM, PVN neurons are activated through the baroreceptor reflex and via neuronal pathways originating at the AP as well (Shan and Krukoff, 2000). In the case of icv injections of ADM, the activation of each division of the PVN can be related to its functional relevance. For example, ADM likely stimulates the HPA axis by activating the mpd division of PVN to release CRF. ADM activates the dp and mpv divisions of the PVN to affect sympathetic functions. ADM also participates in the regulation of the hypothalamo-neurohypophysial system by acting on the mPVN to release the OXY. If ADM can acts directly on the PVN, the presence of ADM peptide (Serrano et al., 2000) and mRNA (Shan and Krukoff, in revision) in the PVN suggests that ADM acts in an autocrine and/or paracrine manner to regulate PVN functions.

It is well established that central NO participates in the regulation of body homeostasis (Krukoff, 1999). Our earlier studies have reported that NO participates in regulating central responses to various stimuli including immune, restraint stress and hypotension (Petrov et al., 1995; Krukoff and Khalili, 1997; Yang et al., 1999; Yang and Krukoff, 2000). Our iv and icv studies now also implicate NO in ADM-induced responses. However, the mechanisms through which NO regulates ADM-induced responses await further investigation.

ADM has been shown to act on the CNS to elicit effects complementary with or opposite to its periphery actions. For example, centrally administered ADM suppresses water (Murphy and Samson, 1995) and salt (Samson and Murphy, 1997) intake, and induces diuretic and natriuretic actions (Israel and Diaz, 2000), which are matched by the diuretic and natriuretic effects in the kidney (Vari et al., 1996). On the other hand, our iv

and icv ADM injections studies show that the hypotensive effects of ADM in the periphery are opposite to its hypertensive effects in the brain. Similarly, we demonstrate that icv injections of ADM increase ACTH levels, in contrast to its direct inhibitory release of ACTH from the pituitary gland (Samson et al., 1995; Parkes and May, 1995). Together, these results suggest that ADM acts on the CNS to counterbalance its peripheral effects in order to restore the homeostatic balance in animals.

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